



UNIVERSITY OF
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Defining the role of microRNA-122 in
the early detection of chemotherapy-
induced hepatotoxicity in the neo-
adjuvant treatment of advanced
colorectal cancer.

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the University of Liverpool for the degree of Doctor of
Medicine

By

Derek McWhirter

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been, nor is currently being presented wholly, or in part, for any other degree or qualification.

Derek McWhirter

- Registrar in General Surgery, Mersey Deanery School of Surgery and Liverpool Hepatobiliary Centre, University Hospital Aintree, Liverpool, UK.
- Clinical Research Fellow, MRC Centre for Drug Safety Science, Institute of Translational Medicine, University of Liverpool, UK.

This research was undertaken at the MRC Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, University of Liverpool under the supervision of;

Dr Neil Kitteringham

- *Senior Lecturer, MRC Centre for Drug Safety Science, University of Liverpool*

Professor Daniel Palmer

- *Chair of Medical Oncology, Department of Molecular and Clinical Cancer Medicine, University of Liverpool.*
- *Honorary Consultant in Medical Oncology, Clatterbridge Cancer Centre, Wirral.*

Mr Hassan Malik

- *Consultant Hepatobiliary and General Surgeon, Liverpool Hepatobiliary Centre, University Hospital Aintree, Liverpool.*

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Abstract

Defining the role of microRNA-122 in the early detection of chemotherapy-induced hepatotoxicity in the neo-adjuvant treatment of advanced colorectal cancer.

Derek McWhirter

Colorectal cancer remains one of the most common cancers in the United Kingdom with around 40,000 new cases being diagnosed each year. Around 25% of patients will have liver metastases at the time of presentation, with up to 50% developing metastases at some point in their life. Advances in surgical technique and developments in chemotherapy have increased the number of patients with advanced disease for whom potentially curative treatment is possible. The use of chemotherapy in a neo-adjuvant setting has improved the outcome for patients with liver metastases who have initially irresectable or borderline disease. Chemotherapy-induced hepatotoxicity affects up to 78% of patients receiving standard chemotherapy for colorectal cancer and can lead to increased morbidity and mortality. Current gold standard serum-based biomarkers of drug-induced hepatotoxicity have their limitations and there remains a need for more sensitive and specific novel biomarkers to detect early hepatotoxicity.

The use of serum-based microRNAs, in particular microRNA-122 (miR-122), a hepatocyte-specific molecule has been proposed as a possible biomarker for chemotherapy-induced hepatotoxicity.

The work described in this thesis assessed the characteristics of serum miR-122 in a healthy human population. It also assessed serum levels of miR-122 in different diseases including primary liver cancer. In order to assess the role of serum miR-122 in chemotherapy-induced hepatotoxicity, a pilot study was carried out in patients receiving chemotherapy for advanced colorectal cancer.

In a healthy human population (n=129) serum levels of miR-122 were measured to investigate the degree of variation and define a normal reference range that could be used to assess changes found in patients with hepatic injury and disease. In addition to miR-122, two endogenous (U6snRNA/let-7d) and one exogenous controls (c.lin-4) were measured. Inter-individual variation was low for miR-122 (CV% 5.21) and also for all three controls (CV% <4%). There was no circadian variation in serum miR-122 (ANOVA p=0.1254). Analysis of intra-patient variation over three consecutive days was similarly low (p=0.66).

In a human population with underlying chronic liver disease (n=90) and primary liver cancer (n=104), serum miR-122 was significantly raised in the both cohorts (p=<0.001) but no difference was seen between the chronic disease and cancer cohorts (p=0.338). Patients with an underlying inflammatory condition had significantly raised serum miR-122 (p=<0.001)

compared to those with underlying cirrhotic or fibrotic change ($p=0.372$). ROC analysis supported this finding (AUC 0.79 vs 0.54).

In a pilot study of serum miR-122 during neo-adjuvant chemotherapy for colorectal cancer liver metastases, 11 patients were recruited. Serial blood sampling during chemotherapy treatment revealed a non-significant rise in miR-122 ($p=0.14$). Clinically insignificant levels of liver toxicity were seen in the ten patients who completed the treatment and had surgery. In those with histology changes known to be associated with chemotherapy, there was a significant rise in serum ALT ($p=0.0082$ and 0.0085) while the miR-122 did not rise significantly ($p=0.053$).

This work confirmed the low variation in serum miR-122 that is a requirement for a novel biomarker. Furthermore, it confirmed the detectable increase of serum miR-122 in patients with liver disease, particularly those with an inflammatory pathophysiology. Finally, in a human model of chemotherapy-related hepatotoxicity, the role of miR-122 remains unclear at present, but the non-significant changes in level related to clinically insignificant liver perturbation compared to the significant changes in ALT suggest that, although more work is required, it may be a valuable biomarker with potential in this field.

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Final thanks must go to my family, in particular my wife Katie, who had to endure two years of a simple surgeon trying to understand science and all the stress that went with it.

This thesis is dedicated to my brother Craig (1984-2008) who will forever remain my best friend and who taught me that you seize every opportunity in life and live each day like it's the last.

Publications

Research Articles

McWhirter D, Kitteringham NR, Park BK, Starkey Lewis P, Goldring CE, Fenwick SW, Malik HZ, Johnson P and Palmer D. MicroRNA-122 may have no role as a serum biomarker for hepatocellular carcinoma. *Manuscript in preparation*.

Dunne DFJ, Gaughran J, Jones RP, **McWhirter D**, Sutton PA, Malik HZ, Poston GJ and Fenwick SW. Routine staging laparoscopy has no place in the management of colorectal liver metastases. *EJSO* 39 (2013) 721-725.

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McWhirter D, Kitteringham NR, Jones RP, Malik HZ, Park BK and Palmer D. Chemotherapy induced hepatotoxicity in metastatic colorectal cancer: A review of mechanisms and outcomes. *Critical Reviews in Oncology/Hematology* 88 (2013) 404–415

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McWhirter D, Starkey Lewis P, Kitteringham N, Antoine D, Pirmohamed M, Goldring C, Park BK, Malik HZ. Defining the role of MicroRNA-122 as a potential circulating biomarker for liver injury and disease. *British Journal of Surgery* 2013;100 (S8):15.

Abbreviations

5-FU	5-fluorouracil
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANOVA	Analysis of Variance
APC (gene)	Adenomatous Polyposis Coli
APC (metabolit	7-ethyl-10-[4-N-(5-aminopentanoic acid)- 1- piperidino]carbonyloxycamptothecin
AST	Aspartate Transaminase
AUC	Area Under the Curve
BER	Base Excision Repair
BRAF	Proto-oncogene B-Raf
CASH	Chemotherapy-Associated Steatohepatitis
CCC	Clatterbridge Cancer Centre
cDNA	Complimentary Deoxyribonucleic Acid
CES 1/2	Carboxylesterase 1/2
CRC	Colorectal Cancer
CRLM	Colorectal (Cancer) Liver Metastases
CT	Computed Tomography
Ct	Cycle Threshold
CT-PET	Computed Tomography combined with Positron Emission Tomography
CV%	Coefficient of Variance
DACH	Diaminocyclohexane
DFS	Disease Free Survival
DHFU	Dihydrofluorouracil
DILI	Drug-Induced Liver Injury
DPD	Dihydropyrimidine Dehydrogenase
EGFR	Epidermal Growth Factor Receptor
EORTC	European Organisation for Research and Treatment of Cancer
FAP	Familial Adenomatous Polyposis
FDG	Fluorodeoxyglucose
FdUMP	Fluorodeoxyuridine Monophosphate
FdUTP	Fluorodeoxyuridine Triphosphate
FLR	Future Liver Remnant
FOLFIRI	Folinic Acid, 5-FU and Irinotecan
FOLFOX	Folinic Acid, 5-FU and Oxaliplatin
FOLFOXIRI	Folinic Acid, 5-FU, Oxaliplatin and Irinotecan
FUTP	Fluorouridine Triphosphate
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma

HCV	Hepatitis C Virus
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
KRAS	Kirsten rat sarcoma viral oncogene
LFT	Liver Function Tests
LOH	Loss of Heterogeneity
LV	Leucovorin
MAPK	Mitogen-Activated Protein Kinase
miRNA	Micro-RNA
MMR	MISMATCH REPAIR
MRI	Magnetic Resonance Injury
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MWA	Microwave Ablation
NAFLD	Non-Alcoholic Fatty Liver Disease
NER	Nucleotide Excision Repair
NICE	National Institute of Clinical Excellence
OS	Overall Survival
PET	Positron Emission Tomography
PFS	Progression Free Survival
PVE	Portal Vein Embolisation
qRT-PCR	Qualative Real Time Polymerase Chain Reaction
RFA	Radiofrequency Ablation
RLUH	Royal Liverpool University Hospital
RNA	Ribonucleic Acid
ROC	Receiver Operator Characteristics
ROS	Reactive Oxygen Species
RR	Response Rate
RT	Reverse Transcription
SOS	Sinusoidal Obstruction Syndrome
TNM	Tumour/Nodes/Metastases
TS	Thymidylate Synthase
UHA	University Hospital Aintree
ULN	Upper Limit of Normal
US	Ultrasound
VEGF	Vascular Endothelial Growth Factor
XELOX	Capecitabine and Oxaliplatin

Chapter 1

General Introduction

1.1 Epidemiology of Colorectal Cancer

Colorectal cancer is the 3rd most common cancer in the United Kingdom accounting for 13% of new cases. It is the 3rd most common cancer in men in the United Kingdom accounting for 14% of new cancers and is the 2nd most common cancer in females accounting for 12% of new cases. In 2009, there were 41,142 new cases in the UK with a male to female ratio of 1.2:1. (Cancer Research UK Statistics 2009)

It is the 2nd most common cause of cancer death accounting for 10% of all cancer death. In 2010 there were 16,013 deaths in the UK from colorectal cancer. The 5-year survival varies with stage.

Duke's Stage at Diagnosis	5-year survival
A	93.2%
B	77.0%
C	47.7%
D	6.6%

Table 1: Survival by Dukes Stage. Adapted from Cancer Research UK Statistics Website.

1.2 Genetics of Colorectal Cancer

The majority (70-85%) of colorectal cancers are sporadic tumours with no obvious hereditary basis to them. The remaining 15-30% of cases may have a major hereditary component to them such as Hereditary Non-Polyposis Colorectal Cancer (HNPCC) or Familial Adenomatous Polyposis (FAP) (1-2). Although they account for only a small percentage of the total number of cases of colorectal cancer, the hereditary tumours have given us the most insight into the molecular pathways associated with the development of these tumours.

1.2.1 The adenoma-carcinoma sequence

Polyps within the colon and rectum are fairly common. Most of them are benign, hyperplastic polyps less than 5mm in size and, as a rule, generally do not develop into cancer. (3) It is the adenomatous polyps (adenomas) that can develop into carcinoma over time. They arise from glandular epithelium within the colon and rectum and develop dysplastic changes that can proceed to malignancy. A large study in the United States estimated that around 25% of the population has polyps by the age of 50, and around 50% have polyps but the age of 70.(4) The National Polyp Study Workgroup, again in the United States, showed that there is an increased risk of developing colorectal cancer in polyps that are not removed. When polyps are removed, the risk decreases. (5) Patients who are genetically predisposed to developing polyps such as those with FAP generally develop colorectal cancer early in their 30's-50's and as such prophylactic colectomy is recommended for these patients. (6; 7)Only a small number of polyps develop into cancer and the progression occurs usually over a period of years to decades. A study in the 1980's estimated that a 1cm adenoma has approximately a 10-15% chance of developing into colorectal cancer in 10 years. (8)

In 1990 Vogelstein and Fearon described a model for the development of a carcinoma from an adenoma explained by the underlying genetics. This model is colloquially known as the “Vogelgram”. (9)



Figure 1: The “Vogelgram” adapted from Fearon et al 1990. It describes the key genetic steps in the development of a colorectal cancer. (9)

1.2.2 APC Gene Mutation

The adenomatous polyposis coli (APC) gene is a tumour suppressor gene that is commonly mutated in colorectal cancer. It encodes a protein thought to have important roles in regulating cell-cell adhesion, cell migration, chromosomal segregation and apoptosis in the colonic crypt. (10) It plays a prominent role in the FAP syndromes and its variants but is also highly prevalent in the sporadic tumours, with 70-80% of colorectal cancers having a mutation leading to inactivation of APC. (10) It is thought that APC mutation is the 1st and rate-limiting step in the adenoma-carcinoma sequence as it has the same frequency of mutation in very small adenomas as it does in advanced carcinoma. (9)

A major suggested role for APC has been its role as a regulator of β -catenin protein in the β -catenin dependent Wnt signaling pathway. When APC is mutated and inactive the normal pathway is disrupted leading to accumulation of β -catenin in the cell, resulting in a downstream effect on proto-oncogenes. (11)

1.2.3 KRAS/BRAF mutation

The Ras family, comprising of KRAS, HRAS and NRAS, is a small family of G-proteins acting as molecular switches downstream of cell wall growth factor receptors. They are often mutated in colorectal cancer. (12) KRAS mutations are also seen in non-dysplastic polyps confirming that although it has a role in the development of colorectal cancer it is not the initiating step. (10) Mutations in KRAS have knock-on effects including on the mitogen-activated protein kinase (MAPK) and PI3K pathways that are involved in proliferation and survival of the dysplastic cells. (10) B-Raf is a protein kinase activated by the Ras proteins is also associated with these pathways and is mutated in around 5-10% of colorectal cancer patients. (13) Disruption in the normal

function of these pathways is thought to be critical in tumour development and as such has been the focus of research into targeted therapy. Currently there is a lot of interest in the use of anti-EGFR monoclonal antibodies such as cetuximab in the treatment of colorectal cancer.

1.2.4 p53 mutations

According to the Vogelgram, the final steps from adenoma to carcinoma are controlled by the p53 tumour suppressor gene. Mutation and inactivation of p53 is thought to stem from loss of heterozygosity (LOH) on chromosome 17q in one allele with a somatic mutation in the remaining gene. This combination is found in around 70% of colorectal cancers. (9; 14; 15) When faced with cellular stress p53 activates cell cycle arrest, apoptosis and antiangiogenesis pathways. When mutated the cell does not perform these functions under maximal stress conditions around the time of transition from adenoma to carcinoma. (10)

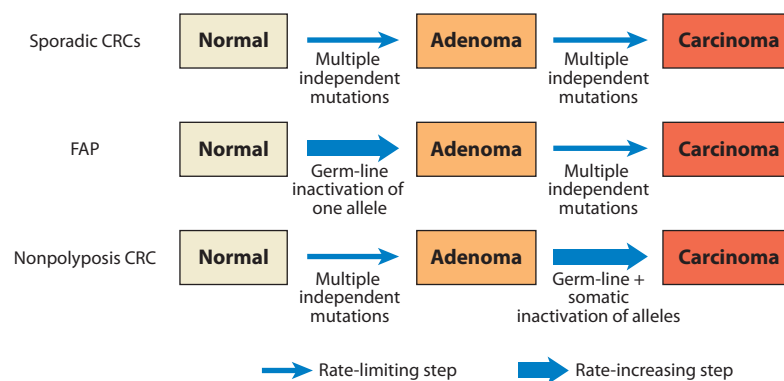


Figure 2: The various rate-limiting and rate-increasing steps involved in the development of colorectal cancer of differing aetiologies. (10)

1.3 Genetic Basis of the Development of Colorectal Liver Metastases

In 1889 the English surgeon Stephen Paget published his seminal “seed and soil” hypothesis to explain the non-random pattern of tumour metastases. The basis of his theory was that certain tumour cells (the seed) have a

specific affinity for certain organs (the soil). He concluded that a metastasis would only develop if the 'seed' and the 'soil' were compatible. (16)

It has been shown that within 24 hours of entering the circulation only 0.1% of tumour cells remain viable and that less than 0.01% survive to produce metastases. (17) Human tumours are made up of a heterogenous population of cells and it is a sub-group of cells within it that have the biological characteristics that give them metastatic potential. (18)

There are a number of rate-limiting steps needed for a tumour to produce a metastasis as depicted in the figure below.

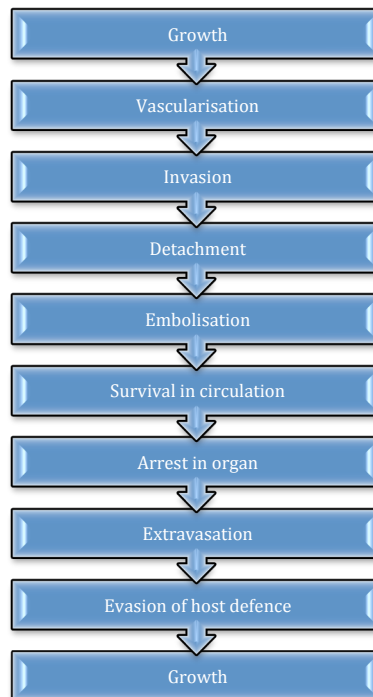


Figure 3: The key steps in the development of a metastasis from a primary tumour. Adapted from Fidler 2003. (16)

Throughout these steps there are a number of complex factors and interactions between the tumour cells and host cells, which determine the fate of potential metastases as, summarised in the table below.

Cell type	Facilitation of metastasis	Inhibition of metastasis
Tumour cells	Production of growth factors and their receptors Production of angiogenic factors Motility, invasiveness Aggregation, deformability Specific cell-surface receptors and adhesion molecules	Antigenicity Inhibitors of angiogenesis Cohesion (E-cadherin) Tissue inhibitors of proteolytic enzymes
Host cells	Paracrine and endocrine growth factors Neovascularization Platelets and their products Immune cells and their products	Tissue barriers Blood turbulence, endothelial cells Tissue inhibitors of proteolytic enzymes Antiproliferative factors Inhibitors of angiogenesis

Figure 4: The regulation of metastases from Fidler 2003. (16)

The microenvironment of different organs attracts different tumours. Endothelial cells in the vasculature of the organs express different cell surface receptors (19) and different growth factors which decide which tumours develop metastases in them. (20)

It has been hypothesised that in the future, therapies targeting metastases should not solely focus on the tumour itself, but in targeting the homeostatic mechanisms that allow and promote growth of the metastasis.

1.4 Staging of Colorectal Cancer

In 1932, Cuthbert Duke, a pathologist working at St Marks Hospital in Harrow, published his work on a staging system for rectal cancer. (21) The tumours were classified into Dukes A (tumour confined to the bowel wall), Dukes B (tumour invading through the serosa) and Dukes C (local lymph node metastases). Over time the classification was subtly altered with Dukes C being divided into C1 (lymph node involvement) and C2 (apical node involvement) to sub-characterise the different outcomes seen. (22) The staging was eventually informally adopted for tumours of the rest of the colon and not just the rectum and the stage Dukes D was also added to signify distant metastases. This stage was not in the original article because as a pathologist, Duke worked only with the resected rectal specimens.

Today, the Dukes system is still in use, but the gold standard, as with most other solid tumours is the TNM system initially described by Pierre Denoix in the 1940's in Paris. This system classified the tumour by the extent of the primary tumour (T), the position of involved lymph nodes (N) and distant metastases (M). The American Joint Committee for Cancer (AJCC) produced updated guidelines every few years and the current guidelines were published as the 7th edition in 2010. (23)

TMN Stage	Stage Description
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in-situ: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades through muscularis propria
T3	Tumour invades through muscularis propria into pericorectal tissues
T4a	Tumour penetrates to the surface of the visceral peritoneum
T4b	Tumour directly invades or is adherent to other organs or structures
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastases in 1-3 regional lymph nodes
N1a	Metastases in 1 regional lymph node
N1b	Metastases in 2-3 regional lymph nodes
N1c	Tumour deposit(s) in the subserosa, mesentery, or non-peritonealised pericolic or peri-rectal tissues without regional nodal involvement
N2	Metastases in 4 or more regional lymph nodes
N2a	Metastases in 4-6 regional lymph nodes
N2b	Metastases in 7 or more regional lymph nodes
M0	No distant metastases
M1	Distant metastases
M1a	Metastases confined to one organ or site
M1b	Metastases in more than one organ/site or the peritoneum

Table 2: The 7th edition of the AJCC TNM staging system for colorectal cancer. Adapted from Obrocea et al. (23)

The complexities of the TNM stage make it difficult to apply to clinical practice in everyday life. For this reason the tumours are grouped into 1 of 4 'Stages' in order that management plans can be made.

Staging of Colorectal Cancer	
Stage 1	Primary tumour into but not through muscularis propria, and no metastases.
Stage 2	Primary tumour grown through to serosa and peritoneal surface but no metastases.
Stage 3	Any size of primary tumour with lymph node metastases.
Stage 4	Presence of distant metastatic disease.

Figure 5: Staging of colorectal cancer.(24)

1.5 Diagnosis and Imaging in Colorectal Liver Metastases

As with other cancers of the gastrointestinal tract, the spread of colorectal cancer is via the portal vein to the liver before entering the systemic circulation. Indeed, the liver may be the only site of spread in 25-40% of patients with metastatic disease. (25; 26). Around 25% of patients will have liver metastases at the time of presentation of the primary, known as synchronous disease, while up to 50% will develop metastases in the liver at some point in their lifetime. (27-29) Colorectal cancer is one of few cancers where metastatic spread is not an absolute contraindication to potentially curable disease. Early diagnosis and good follow-up with quality imaging is essential if good outcomes are to be achieved.

1.5.1 Ultrasound

Ultrasound (US) is a widely available, non-invasive modality that does not involve the use of ionising radiation. Liver metastases tend to be hypoechoic on US, but have generally non-specific features making it harder to accurately categorise them. There are also several technical limitations with ultrasound. The main two are the skill and experience of the radiographer and the body habitus of the patient making interpretation difficult. In order to increase the sensitivity of the test, contrast ultrasound is becoming more readily available. This utilises intravascular microbubble contrast agents to enhance the texture of the liver during the arterial phase. This makes small hypovascular metastases easier to diagnose. Studies have shown that the

use of contrast enhancement has increased the sensitivity by around 20%(30) and that around 97% of CT detected lesions are also seen on contrast US. (31)



Figure 6: US showing liver metastases. The image on the left is unenhanced with no contrast in the portal vein (double arrow). After contrast enhancement on the right with contrast in the portal vein (double arrow), 2 liver metastases are now clearly visible (single arrows). (31)

1.5.2 Computed Tomography

Computed tomography (CT) is the most commonly used modality for the detection, characterization and follow-up of liver lesions including colorectal liver metastases. It has a detection sensitivity of between 60-90% and has the added advantage of being able to scan the full thorax, abdomen and pelvis to fully stage the disease and detect extra-hepatic disease. (32)

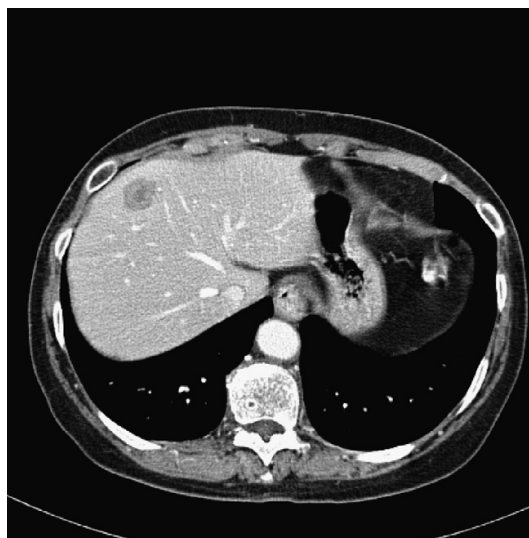


Figure 7: Colorectal liver metastases as seen on CT in the portal venous phase. (32)

1.5.3 Magnetic Resonance Imaging

If considered resectable further imaging is required. NICE guidelines issued in November 2011 (CG131) does not give specific guidance on which extra modalities are required but states that it is at the discretion of the individual MDT. The most commonly used are magnetic resonance imaging (MRI) and positron emission tomography (PET). MRI is used to fully characterise the metastases. (33) It can give more information and there is increased sensitivity after the use of contrast agents. (34).

1.5.4 Positron Emission Tomography

PET involves the uptake of 18-fluorodeoxyglucose (FDG) radiotracer by metabolically active tissue. The FDG is transported into cells and undergoes phosphorylation by the enzyme hexokinase. It cannot then be metabolised by the glycolytic pathway and accumulates in the cells. Tumours tend to be more metabolically active and therefore more tracer is taken up by these tumours creating a “hot-spot” on the scan image. (32; 35). It is commonly combined with CT scanning (CT-PET) to give more accurate anatomical information. CT-PET has the advantage of being able to detect lower volume disease than plain CT (36) as the metabolic abnormalities of the tumour can often be detected before gross anatomical changes are seen. (32) CT-PET has actually been shown to have a major role in altering management in over a quarter of patients where it is used. (37)

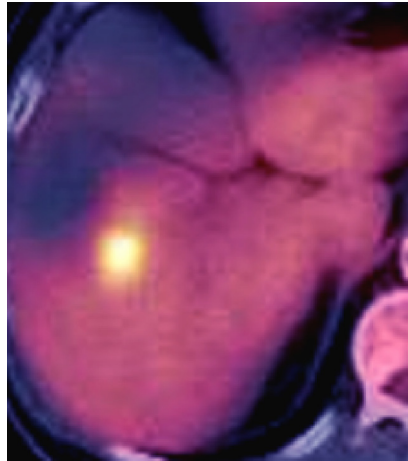


Figure 8: A “hot-spot” in the liver on CT-PET detecting a metastasis. (32)

1.6 Surgical Resection/Ablative Management of Colorectal Liver Metastases

Surgery for colorectal liver metastases has made considerable progress over the past few decades. In the early to mid-90’s surgery with curative intent was only considered for patients meeting the most stringent of criteria. (38) These were:

- 1-3 unilobar metastases with preferably metachronous presentation
- resectable with a generous margin (R0)

Changes in chemotherapy and surgical innovation have changed the landscape and current guidance is that all patients fit enough for surgery who could have all their disease removed with adequate future remnant liver (FRL) volume should be considered for surgery. (24) At a minimum, a future liver remnant of around 25% (2 Couinaud segments) are required to maintain adequate function. If there is impaired hepatic function, then a greater FRL is required and the extent of chemotherapy toxicity must be considered. If the planned resection is extensive and a borderline FRL is predicted, it is possible to perform portal vein embolisation (PVE) before resection in order to induce hypertrophy of the FLR to allow the resection to proceed safely. (39) Recently the need for microscopically clear margins

(R0) has also been challenged. Pawlik et al showed that although the difference in survival outcome was better in R1 resections (microscopic evidence of tumour at the resection margin) than R2 resections (macroscopic evidence of tumour at the resection margin), the outcomes for R1 and R0 (no microscopic tumour at the resection margin) were similar. (40)

An alternative to surgical resection is ablation of the tumour. There are multiple modalities of this including radiofrequency ablation (RFA) and microwave ablation (MWA). They can be performed at the time of laparotomy or percutaneously, which has the benefit of not requiring surgery. Despite much interest, there remains little high quality evidence for its use. The American Society of Clinical Oncology (ASCO) advised in 2009 that in the absence of good evidence, that surgery remains the gold standard. (41) The use of percutaneous ablative techniques is often reserved for unfit patients with small tumours.

To date, the only randomised trial is the CLOCC trial (EORTC 40004) where patients with irresectable disease were randomised to receive chemotherapy +/- RFA (n=59/60). Median follow-up was 4.4 years with 30 month overall survival favouring the RFA and chemotherapy arm (61.7% vs 57.6%) with a median progression free survival of 16.8 months vs 9.9 months (p=0.025). A Cochrane Review in 2012 found that the evidence for the use of ablation was too sparse to recommend its use in radical treatment of metastases. (42)

1.7 Chemotherapy in Colorectal Cancer

Around 20-25% of patients will present with synchronous disease and a further 20% will develop metastases at some point after resection of the colorectal primary. The National Institute for Clinical Excellence (NICE) has produced guidance that states that adjuvant chemotherapy should be considered the standard of care in all high-risk Stage II and all Stage III

cancers following resection of the primary tumour. (24) The use of chemotherapy in operable Stage IV disease is less well defined at present.

In the past decade there has been a significant increase in the survival of patients with Stage IV colorectal cancer. A multi-center review suggested that improvement in survival of patients diagnosed with colorectal cancer was due to more successful treatment of metastatic disease by a combination of surgery and chemotherapy. (43) In the early 21st century, only 5-fluorouracil was available for the treatment of colorectal cancer. Recently there have been significant developments and multiple agent are available. Currently the mainstay of treatment is with 5-fluorouracil, leucovorin, oxaliplatin, irinotecan, capecitabine and the monoclonal antibodies cetuximab and bevacizumab. They are rarely given as monotherapy and are usually combined as FOLFOX (5-FU, leucovorin and oxaliplatin), FOLFIRI (5-FU, leucovorin and irinotecan) or XELOX (capecitabine and oxaliplatin) +/- antibodies.

1.7.1 Adjuvant Chemotherapy for Advanced Colorectal Cancer

In a large phase III trial, de Gramont et al randomised 420 patients with unresectable colorectal liver metastases to receive 5-FU +/- oxaliplatin. There were significant increases in both tumour response rate (50.7% vs 22.3%, $p=0.0001$) and progression free survival (9.0 vs 6.2 months, $p=0.0003$) in the oxaliplatin-receiving group. However, there was no difference in overall survival (16.2 vs 14.7 months, $p=0.12$). (44) In Europe, several large-scale trials were then performed to assess the role of adjuvant chemotherapy in resected Stage II and III colorectal cancer. The MOSAIC study included 2,246 patients who received 5-FU and leucovorin +/- oxaliplatin following resection. This showed an increase in disease free survival (DFS) and overall survival (OS) in Stage III disease (HR 0.8. 95% C.I. 0.65-0.97, $p=0.023$) but no benefit in Stage II. (45) Meanwhile, the Pan-European Trials in Alimentary Cancer (PETACC-3) study involved 2,094 patients randomised to either receive 5-FU/LV +/- irinotecan. No difference

was seen in either 5-year DFS or OS. (46) These trials have led to the use in the United Kingdom of oxaliplatin as the first line agent in advanced colorectal cancer, with irinotecan reserved for second line. In the United States, the Irinotecan Study Group performed a large Phase III randomised trial to assess the effect of irinotecan in palliative patients. 683 patients were randomised to either 5-FU/LV alone; irinotecan alone; or 5-FU/LV + irinotecan. The addition of the irinotecan to the 5-FU/LV resulted in significant increases in PFS (7.0 vs 4.3 months, $p=0.004$), tumour response (39 vs 21%, $p<0.001$) and overall survival (14.8 vs 12.6 months, $p=0.04$). (47) On the basis of this, irinotecan is used as the first line agent in the United States.

1.7.2 Adjuvant chemotherapy for resectable colorectal liver metastases

The FFCD AURC 9002 trial randomised 173 patients with completely resected (R0) hepatic metastases from colorectal cancer to follow-up with observation or 6 months of adjuvant chemotherapy with 5-FU/LV. This showed an increase in 5-year DFS in the chemotherapy group (33.5% vs 26.7%, $p=0.028$) and a non-significant trend towards overall survival (51.1% vs 41.1%, $p=0.12$). (48) The EORTC 0923 trial attempted a similar model but it too was underpowered and no significant differences were shown. A later paired analysis of both trials confirmed a trend of increased overall survival but did not reach significance (62.2 vs 47.3 months, $p=0.095$). (49)

1.7.3 Neo-adjuvant chemotherapy for colorectal liver metastases

In 2006 the MAGIC trial firmly defined the role of neo-adjuvant chemotherapy in the management of gastro-oesophageal adenocarcinoma. (50) There has also been increasing interest the potential role for neo-adjuvant chemotherapy in other gastrointestinal adenocarcinomas.

The potential benefits include reducing tumour volume allowing for the use of parenchymal sparing surgery, the potential effect on micrometastases reducing the risk of recurrence and allowing for observation of response to allow more personalized therapy. Rene Adam and colleagues, who in 2004 retrospectively analysed the outcome of 131 consecutive patients who underwent hepatectomy following neo-adjuvant chemotherapy, have investigated the latter extensively. Following chemotherapy 58 patients (44%) had evidence of disease response, 39 (30%) had no response and 34 (26%) had evidence of disease progression. 5-year survival was higher in those who responded with those who progressed having the lowest survival (37% vs 30% vs 8%, $p < 0.0001$). Disease free survival followed a similar pattern (21% vs 20% vs 3%, $p = 0.02$) (51) This study highlighted the poor outcome of those with aggressive disease, namely those with greater than 4 metastases that progressed on chemotherapy paving the way for these patients to receive second line therapy rather than surgical resection.

There were also some concerns about neo-adjuvant chemotherapy. The primary concerns were the difficult management of the complete response and whether it was safe to give chemotherapy and then operate. Several small studies looked to address this issue. They confirmed that the chemotherapy was generally well tolerated with an acceptable peri-operative morbidity of between 21-50% and that there was 0% operative mortality. (52-54)

A large retrospective study of 1474 patients who either underwent surgery alone ($n=169$) or surgery with peri-operative chemotherapy ($n=1302$) showed that there was an increased number of post-operative complications in the chemotherapy group (37.2% vs 24%, $p=0.006$). In the final analysis, neo-adjuvant chemotherapy did not lead to any increase in overall survival. (55)

The international multi-centre EORTC 40983 (EPOC) trial was designed to try and finally answer the question about the role of neo-adjuvant and peri-operative chemotherapy in resectable colorectal liver metastases. (56) 364 patients with resectable disease were randomised to either surgery alone or 12 cycles of peri-operative FOLFOX (6 pre and 6 post). The primary

endpoint was 3-year progression free survival with secondary endpoints of response rate, safety and overall survival. 94% of those in the chemotherapy arm received at least one pre-operative cycle with 78.6% receiving the full 6 cycles. Within the chemotherapy arm, 8 patients (5%) did not have a resection. 4 of them were found to have new disease and 4 had progressed while on chemotherapy. There was a high dropout rate after the surgery. Only 115 patients (63.2%) in the chemotherapy arm had any post-operative chemotherapy with only 43.9% receiving the full 6 cycles. At analysis, it was shown that of those patients who successfully underwent liver resection, the 3-year PFS was significantly higher in the chemotherapy arm than the surgery alone arm (36.2% vs 28.1%, HR 0.77, 95% C.I. 0.6-1.0, $p=0.041$). There was also a trend towards longer PFS in the intention-to-treat analysis (35.4% vs 28.1%, HR 0.79, 95% C.I. 0.62-1.02, $p=0.058$). Following the publication of this data, the study was used to define the standards of care in many units, particularly in the United Kingdom. (57; 58) More recently longer-term follow-up from the study was presented at the American Society of Clinical Oncology Meeting in 2012. With a median follow-up of 8.5 years, it was shown that nearly half the patients had died from cancer related causes. No benefit was seen in overall survival (HR 0.87, 0.66-1.14, $p=0.303$) although the study was not powered for this secondary endpoint. There was a non-significant increase in 5-year and overall survival in the chemotherapy arm (63.7% vs 55%) (187).

Further trials are ongoing to clarify the role for chemotherapy in resectable disease. In the United States, the NSABP Phase III trial is comparing FOLFOX and FOLFIRI in liver only resectable disease treating with peri-operative chemotherapy (6 cycles pre- and post-resection), while in Europe the Phase II EORTC 40091 is a 3-arm study with adjuvant chemotherapy for resectable disease. The use of cetuximab in resectable disease is also being studied in the New EPOC study, which has closed early after interim data analysis and is currently being prepared for reporting.

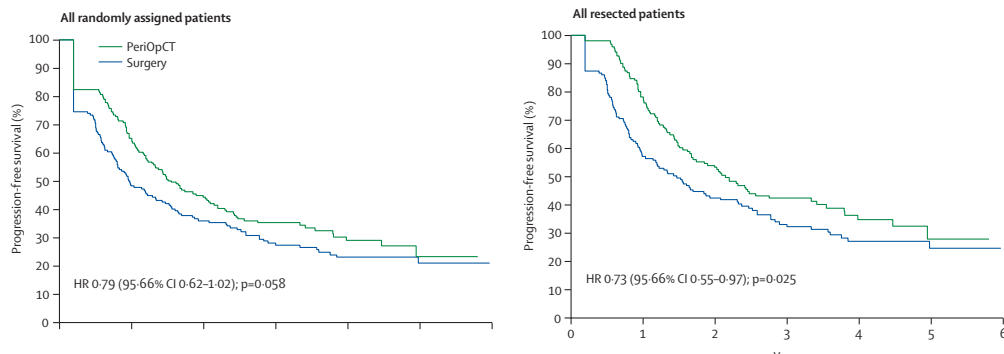


Figure 9: Progression free survival of intention-to-treat and all resected patients in the EORTC 40983/EPOC trial. There was a significant increase in PFS in the chemotherapy arm but at median follow-up of 8.5 months there was no significant increase in overall survival. (56)

1.7.4 Chemotherapy for irresectable disease

It is recognised that there is a group of patients who are initially unresectable at presentation but may become resectable after a response to chemotherapy. (59) The rates of conversion to resectable disease vary considerably in the published literature and rates of 6-60% are often quoted. (60) Adam et al showed that patients who underwent hepatectomy following conversion chemotherapy had a 5-year survival rate of 35-50%, which is equal to those who were resectable at presentation. (61; 62) Folprecht et al performed a systematic analysis of 5 prospective and 1 retrospective studies that showed a strong correlation between higher response rates with chemotherapy and conversion to resectable disease and resection. (63) Increasingly aggressive chemotherapy regimes have been used to achieve higher resection rates. (64)

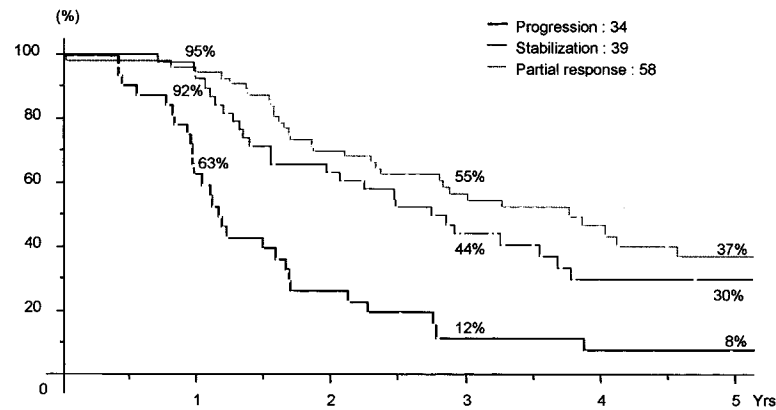


Figure 10: Overall survival in patients undergoing hepatectomy following neo-adjuvant chemotherapy for initially unresectable disease. (61)

The GERCOR study by Tournigand et al in 2004 compared the use of FOLFOX and FOLFIRI in unresectable disease. Patients were randomised to receive either FOLFOX or FOLFIRI until progression of disease or toxicity at which point they were switched to receive the other regime. Response rates of 54-58% were seen in metastases at any site. There was no difference in the PFS in either group as a first line, but in those who switched to FOLFOX increases in PFS were seen (4.2 vs 2.5 months, $p=0.003$). There was no difference seen in overall survival. Resection was achieved in 9% (10 patients) who received FOLFIRI initially followed by FOLFOX and in 22% (24 patients) in those who initially received FOLFOX followed by FOLFIRI. A complete resection (R0) was achieved in 7-13%. (65; 66) Following this work a major systematic review of irinotecan and oxaliplatin was published by the UK Health Technology Assessment Agency. It concluded that resection rates in FOLFIRI were 9-35% and in FOLFOX 7-51%. (67) These studies further confirmed the position of using FOLFOX as first-line therapy in the United Kingdom.

The Gruppo Oncologico Nord Ovest (GONO) performed a randomised Phase III trial to investigate the use of triplicate therapy. 244 patients were randomised to receive either FOLFOXIRI or FOLFIRI. A good response rate was seen with the triplicate therapy, with the R0 resection rate in patients with liver only metastases being 36% as opposed to 12% with FOLFIRI ($p=0.017$). (68) However, there were significant increases seen in side-

effects, particularly peripheral neurotoxicity (0% vs 19%, $p < 0.001$) and neutropenia (28% vs 50%, $p < 0.001$). Due to the increased side-effect profile, triplicate therapy is not used routinely in the first line treatment.

1.8 Biological Agents in Metastatic Colorectal Cancer

The monoclonal antibodies to epidermal growth factor receptor (EGFR), cetuximab (Erbix[®]) and panitumumab (Vectibix[®]), and to vascular endothelial growth factor (VEGF), bevacizumab (Avastin[®]) have recently been the subject of much interest in the treatment of irresectable metastatic colorectal cancer.

These therapies do however have their limitations. It has been shown that patients with a mutation in the KRAS (Kirsten rat sarcoma) oncogene are resistant to treatment with anti-EGF monoclonal antibodies. (69) Currently as the KRAS status of the primary tumour is similar to the metastases, testing of the paraffin-embedded primary tumour can be used to predict if there is any role for anti-EGF therapy in an individual patient. (70)

1.8.1 Cetuximab

Tabernero and colleagues performed a Phase II trial assessing the potential for a role of cetuximab in addition to standard chemotherapy in unresectable metastatic colorectal cancer. In the clinical arm of the study 43 patients received cetuximab in addition to FOLFOX. The overall tumour response rate was 72% and median overall survival was 30 months. 10 patients (23%) were downstaged enough to be considered suitable for resection. Importantly, the drug was well tolerated and caused no extra side effects. (71)

The BOND study randomised 329 patients who had previously been treated with irinotecan to therapy with cetuximab +/- irinotecan. The combination group showed significantly higher tumour response rate (22.9% vs 10.8%,

$p=0.007$) and a non-significant increase in median survival (8.6 vs 6.9 months, $p=0.48$). (72)

The OPUS trial was a Phase II trial comparing FOLFOX +/- cetuximab in 337 patients with unresectable colorectal liver metastases. Higher response rates were seen in the cetuximab group in keeping with other trials (45.6 vs 35.7%).

The EPIC trial was a large randomised Phase III of 1298 patients who had failed therapy with oxaliplatin. Patients were allocated to receive irinotecan +/- cetuximab. Significant increases in tumour response rate (16.4% vs 4.2%, $p<0.0001$) and progression free survival (4.0 vs 2.6 months, $p<0.0001$) were seen with cetuximab therapy. (73)

The CRYSTAL trial randomised 1217 patients to receive either FOLFIRI ($n=609$) or FOLFIRI + cetuximab ($n=608$). In keeping with previous studies, patients receiving cetuximab had a significant increase in tumour response rate (46.9% vs 38.7%, $p=0.005$) and progression free survival (8.9 vs 8.0 months, $p=0.036$). (69) A pooled analysis of the results of the OPUS and CRYSTAL trials to give more power showed significant increases in overall survival, response rate and progression free survival in the cetuximab groups. (74)

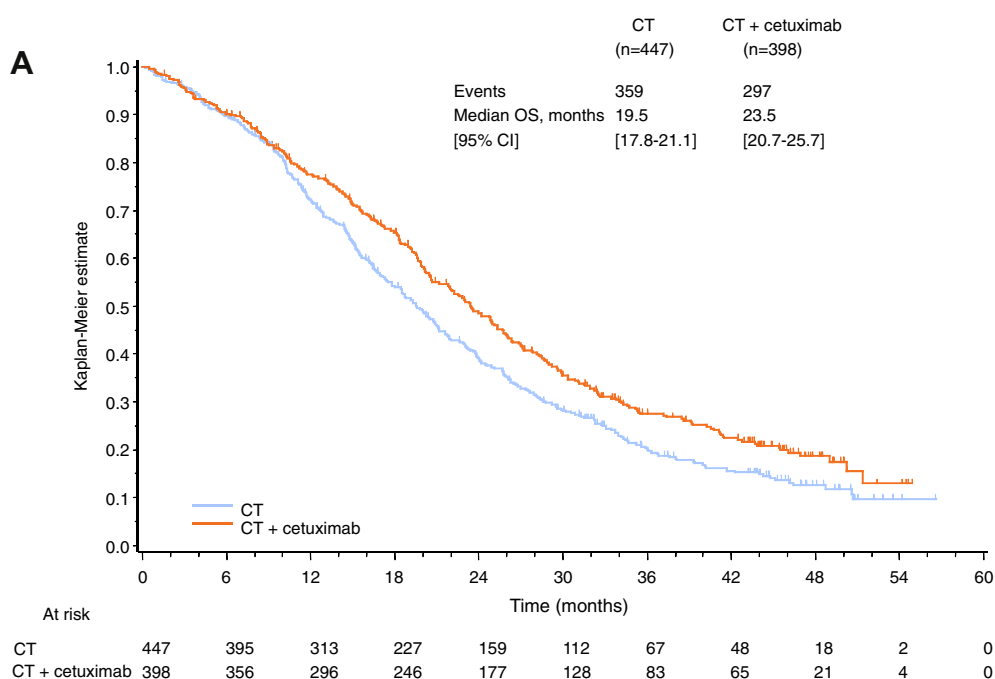


Figure 11: Overall survival in pooled analysis of the OPUS and Crystal trials. Those receiving cetuximab had a significantly increased OS ($p=0.0062$). (74)

A more recent Phase II trial (CELIM) comparing the use of cetuximab in combination with either FOLFOX or FOLFIRI reported high response rates of 68% and 57% respectively. There was no non-cetuximab group in this study which makes the results harder to interpret. (75) The UK MRC COIN trial was a large (n=1630) study comparing the use of cetuximab in combination with FOLFOX. Although there was an increase in response rate, no difference was seen in overall survival meaning they could not recommend the use of cetuximab in first line therapy. (193)

1.8.2 Bevacizumab

In 2004, Hurwitz et al published the results of a large Phase III trial comparing treatment of metastatic colorectal cancer with irinotecan, fluorouracil and leucovorin +/- bevacizumab. 813 patients were randomised with significant increases in median survival (20.3 vs 15.6 months, $p < 0.001$), progression free survival (10.6 vs 6.2 months, $p < 0.001$) and tumour response rate (44.8 vs 34.8 months, $p = 0.004$). (76) Increased side-effects were seen in the bevacizumab group but with no additional mortality or increased drop-out.

The Tree-1 and Tree-2 studies compared various regimens of oxaliplatin with or without the addition of bevacizumab. The combination of FOLFOX and bevacizumab was associated with an increased response rate (11%) and median overall survival of 7 months. (77)

The N016966 trial was a large Phase III trial comparing FOLFOX/XELOX with either bevacizumab or placebo. The primary endpoint was progression free survival where a significant difference was seen in the bevacizumab group (9.4 vs 8.0 months, $p = 0.0023$). There was a non-significant increase seen in overall survival but no difference at all in response rate. (78)

The recent BOXER trial was a small study of 46 patients receiving XELOX and bevacizumab. They obtained a conversion rate to resectability with no unexpected toxicity in 12 of 30 patients initially unresectable (40%). (79)

Adjuvant Chemotherapy for Colorectal Cancer		
Trial/Author	Agent	Outcome
MOSAIC	5-FU+/- oxaliplatin	Increase in DFS and OS in Stage III cancer with oxaliplatin
PETACC-3	5-FU+/- irinotecan	No difference in groups
Adjuvant Chemotherapy for Colorectal Liver Metastases		
Trial/Author	Agent	Outcome
FFCD AURC 9002	5-FU or observation	Increase in 5-year DFS in treatment group
EORTC 0923	5-FU or observation	No difference (under powered)
Neo-adjuvant Chemotherapy for Colorectal Liver Metastases		
Trial/Author	Agent	Outcome
EORTC 40983	Surgery+/- FOLFOX	Increased 3-year PFS. No OS benefit in chemotherapy arm
Chemotherapy for Irresectable Disease		
Trial/Author	Agent	Outcome
De Gramont	5-FU+/- oxaliplatin	Increased tumour RR and PFS in oxaliplatin group
GERCOR	FOLFOX or FOLFIRI (cross-over)	No difference in PFS Increase in PFS in those switched to 2 nd -line FOLFOX Higher resection rate with FOLFOX
GONO	FOLFIRI or FOLFOXIRI	Higher response and resection rate with triple-therapy
Irinotecan Study Group (USA)	5-FU+/- irinotecan or irinotecan alone	Increase PFS, RR and OS in combination group
Biological Agents in Metastatic Colorectal Cancer		
Trial/Author	Agent	Outcome
Tabernero	FOLFOX plus cetuximab	RR of 72%. Drug well tolerated.
BOND	Irinotecan +/- cetuximab	Increased RR with cetuximab
OPUS	FOLFOX +/- cetuximab	Increased RR with cetuximab
EPIC	Irinotecan +/- cetuximab	Increased RR and PFS with cetuximab
CRYSTAL	FOLFIRI +/- cetuximab	Increased RR and PFS with cetuximab
Hurwitz	FOLFIRI +/- bevacizumab	Increased median survival, PFS and RR with bevacizumab
Tree-1/Tree-2	Oxaliplatin +/- bevacizumab	Increased RR with bevacizumab and FOLFOX
NO16966	FOLFOX/XELOX + bevacizumab or placebo	Increased PFS with bevacizumab
COIN	FOLFOX +/- cetuximab	Increase RR with cetuximab but no increase OS

Table 5: Summary of key chemotherapy trials. RR=tumour response rate, PFS= progression-free survival, DFS= disease-free survival, OS= overall survival.

1.9 Assessment of Response to Chemotherapy

In patients receiving chemotherapy in the neo-adjuvant setting, it is important that there is an accurate assessment of how successful the therapy has been. As with most solid organ tumours, the response can be defined pre-operatively by imaging, or post-operatively by histological examination of the specimen.

1.9.1 Radiological Response

Multiple different scoring systems to predict radiological response have been previously described. To overcome the difficulty of multiple systems in a multi-national task group was set up in 1994 including the European Organization for Research and Treatment of Cancer (EORTC), the National Cancer Institute (NCI) of the United States and the National Cancer Institute of Canada Clinical Trials Group. In 2000 they published their guidelines. The Response Evaluation Criteria in Solid Tumours (RECIST) criteria was intended to standardise estimation of response rate to chemotherapy. (80) This scoring system uses the size of the lesions pre- and post-chemotherapy to define the degree of response.

Response	Change in size of lesion
Complete	Lesions disappear
Partial	>30% reduction
Stable	No change
Progressive	>20% increase

Table 3: RECIST criteria for tumour response to chemotherapy

The criteria were updated in 2009 to Version 1.1 that were essentially the same but used different size criteria for selecting target lesions.

It is relatively easy to quantify response based on radiology, but it has been much harder to correlate that response with the degree of pathological response seen when the resected specimen is subjected to histological examination. (81) In a study by Benoist et al in 2006, they compared patients with a complete radiological response with the histological findings. In all, they showed that 83% of patients with a complete radiological response had residual, viable macroscopic or microscopic disease in the resected specimen. (82) It is clear that radiology alone is not reliable and this has led to a difficult clinical situation where uncertainty exists over the appropriate management of patients whose disease has had a complete radiological response to chemotherapy.

1.9.2 Pathological Response

It has long been accepted that patients who exhibit a good response to chemotherapy have a better long-term outcome and increase overall survival. (83) A retrospective review by Blazer et al from the MD Anderson Cancer Centre sought to define survival based on response to chemotherapy. (84) Of the 271 patients whose histology was reviewed, 25 (9%) had a complete pathological response, 97 (36%) had a major response and 149 (55%) had a minor response. The 5-year survival rates are shown in Table 4.

Response to Chemotherapy	Definition of Response	5-year overall survival
Complete	No residual cancer cells	75%
Major	1-49% residual cancer cells	56%
Minor	>50% residual cancer cells	33%

Table 4: 5-year survival rates by pathological response to chemotherapy. (84)

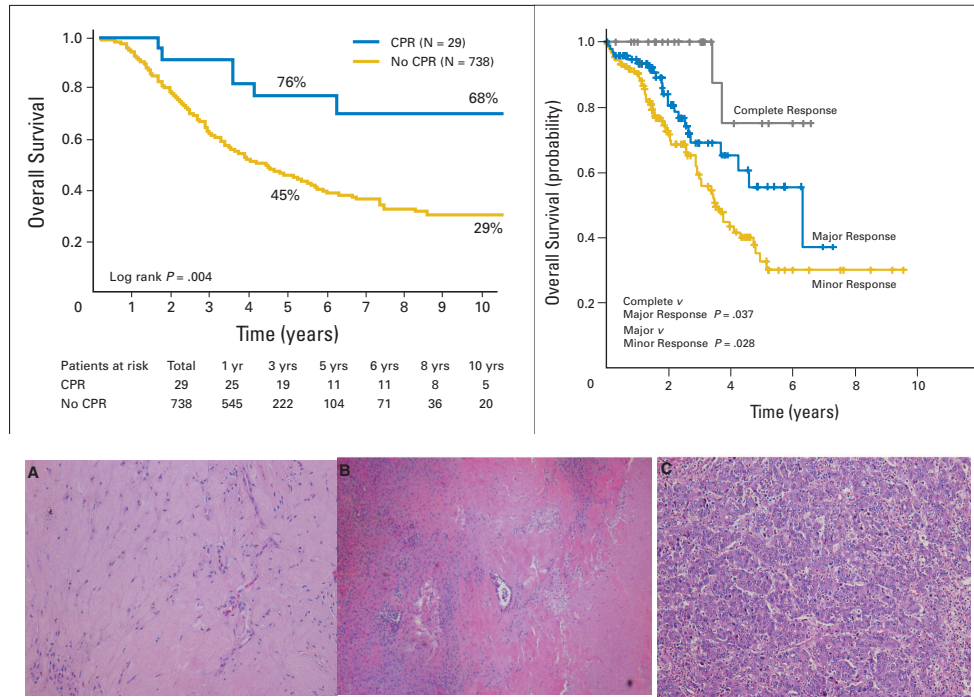


Figure 12: Kaplan-Meier curves showing overall survival by pathological response to chemotherapy and histology of (A) complete response, (B) major response and (C) minor response. (84; 85)

It was clearly shown that there was a significant correlation between pathological response rate and overall survival. This finding was confirmed in a European paper in which Adam et al found a 76% 5-year survival in those with a complete response to chemotherapy compared to 45% in those without. (85)

These studies highlight the potential benefit of neo-adjuvant systemic chemotherapy for those patients with colorectal liver metastases, particularly those who have a good response to treatment.

1.10 Mechanism of Action of Chemotherapeutic Agents

1.10.1 5-Fluorouracil

5-Fluorouracil (5-FU) is a fluoropyrimidine anti-metabolite. Its structure is an analogue of uracil with the substitution of a fluorine atom in place of

hydrogen at the C5 position. It enters cells via a facilitated transport mechanism. Its anti-tumour properties were first developed in the 1950's following work on hepatomas in rats. (86) Its cytotoxicity derives from the misincorporation of fluoronucleotides into the RNA and DNA of host cells and from the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS). (87)

5-FU is rapidly metabolised inside cells to three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP); fluorodeoxyuridine triphosphate (FdUTP); and fluorouridine triphosphate (FUTP). The rate-limiting step in 5-FU catabolism is the enzyme dihydropyrimidine dehydrogenase (DPD) that converts 5-FU to dihydrofluorouracil (DHFU). About 80% of this process occurs in the liver due to the high levels of DPD expressed there.

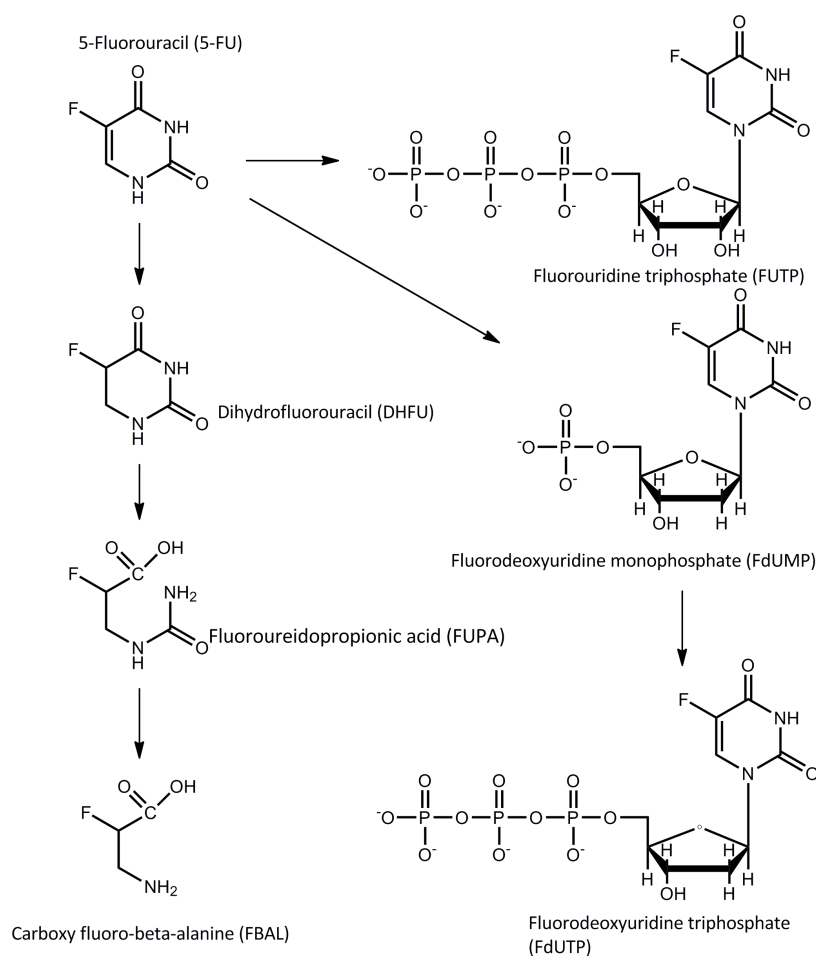


Figure 13: The metabolism of 5-Fluorouracil

The enzyme thymidylate synthase (TS) is essential in producing thymidylate for DNA replication and repair. It involves the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with a reduced folate, CH₂THF, as the methyl donor. FdUMP, as a metabolite of 5-FU, in conjunction with CH₂THF, forms a suicide blockade of the nucleotide binding site on thymidylate synthase preventing dUMP from binding and being converted to dTMP. The resulting reduction in the amount of thymidylate available causes cell death by reducing the amount of thymine available and causing a thymineless cell death. The function of the enzyme thymidine kinase (TK) to produce thymidylate has been suggested as a potential source of resistance to 5-FU. (87) The reduced folate, leucovorin, is often given in combination with 5-FU to help increase its potency.

The remaining two active metabolites, FUTP and FdUTP have been implicated in cell death by causing direct damage to DNA and RNA by misincorporation into them causing disruption of the strands. (88, 89) The DNA repair enzyme uracil-DNA-glycosylase (UDG) is ineffective due to the levels of metabolites and this leads to further DNA strand damage and ultimately cell death. (90)

1.10.2 Irinotecan

Irinotecan is an analogue of camptothecin, a naturally occurring cytotoxic extracted from *Camptotheca acuminata* and was developed as an anti-cancer drug in the early 1970's. (91) It is a Topoisomerase 1 inhibitor that binds to the DNA/Topoisomerase 1 complex during DNA replication preventing resealing of single strands during DNA coiling and uncoiling. This results in double-strand DNA breaks, leading to apoptotic cell death. (92)

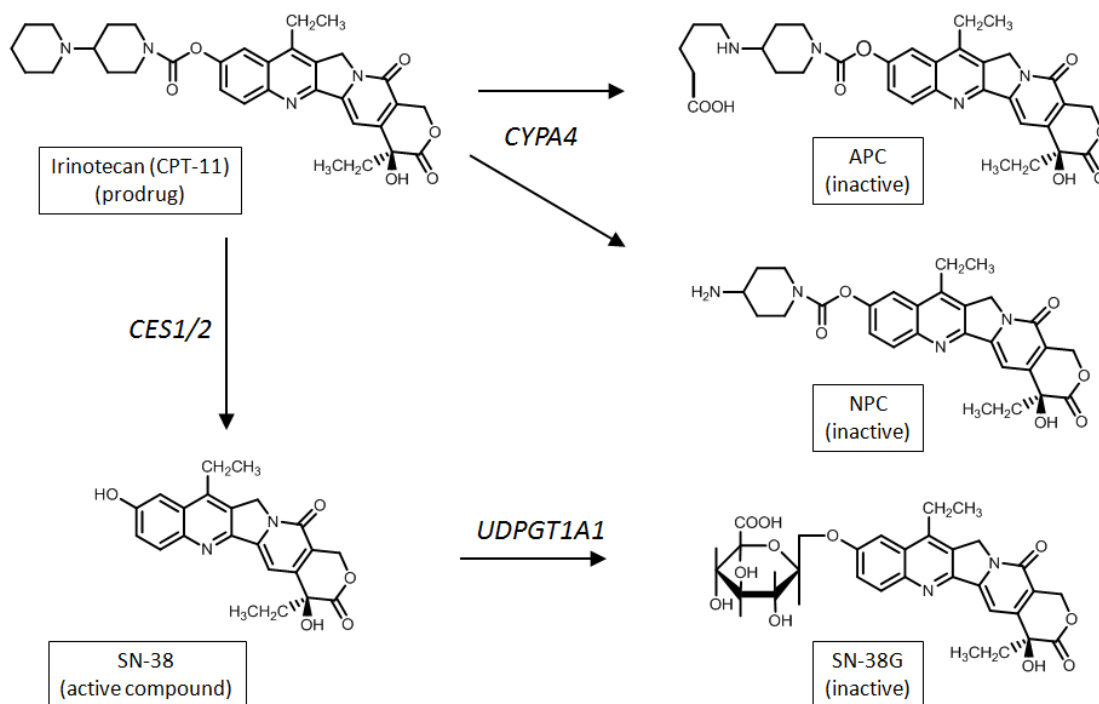


Figure 14: The metabolism of Irinotecan

Irinotecan is a pro-drug that is converted to the active metabolite SN-38 via human carboxylesterases CES1 and CES 2. The cytochrome P450 enzyme CYP3A4 also converts irinotecan to its inactive metabolite APC. The active SN-38 is inactivated to SN-38G by glucuronidation via the enzyme UDP-glucuronosyltransferase. The most active form is the UGT1A1 enzyme and interindividual variation in expression of this enzyme, due a relatively common polymorphism, has been linked to differing responses to, and toxicity from, the drug. (93) Its major toxicity and side effects are diarrhoea and neutropenia although hepatotoxicity is increasingly recognised.

1.10.3 Oxaliplatin

Platinum-based chemotherapy agents have been in use for over 30 years. Several analogues have been developed to overcome issues surrounding drug resistance and toxicity. Oxaliplatin, a platinum-based compound containing a diaminocyclohexane (DACH) ring, has a wide spectrum of anti-

tumour activity and is currently used as first-line treatment for the management of metastatic colorectal cancer.

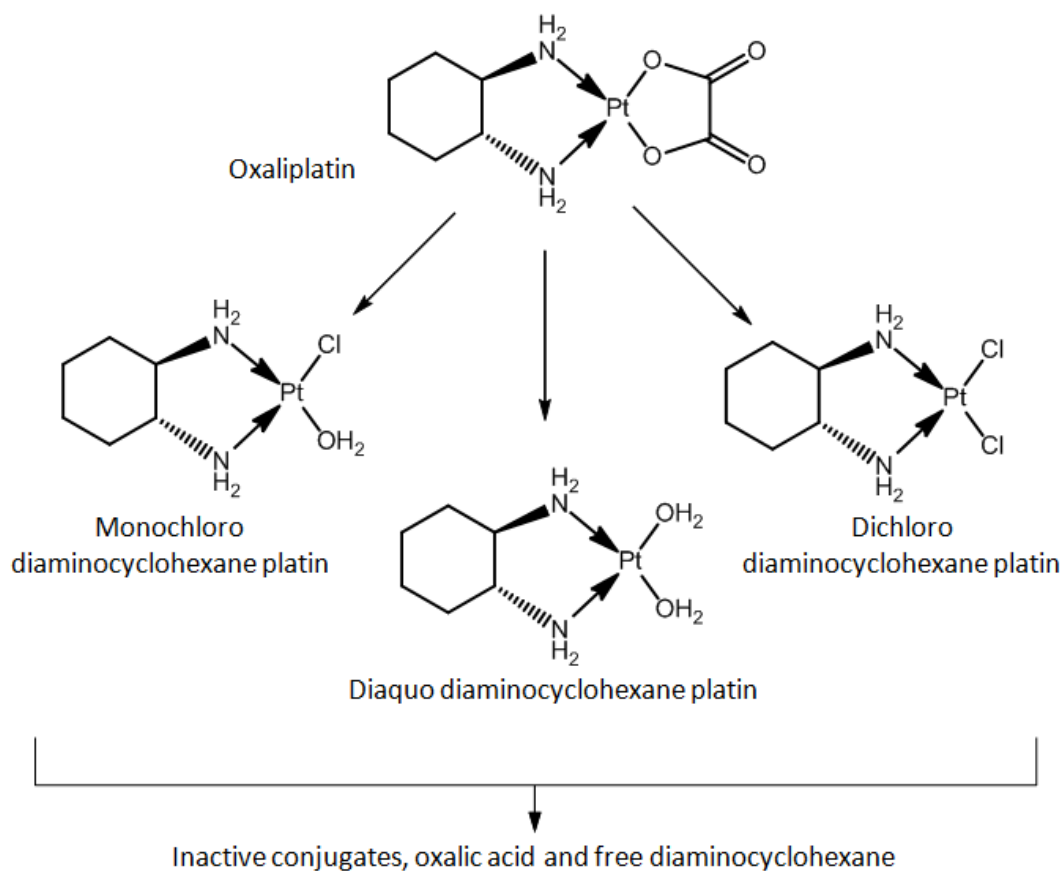


Figure 15: The metabolism of Oxaliplatin

The cytotoxic activity of oxaliplatin is from direct DNA damage. It undergoes biotransformation *in vivo* into a number of metabolites that all contain a DACH ring. (94) DACH-Pt DNA adducts are formed by cross-linking of the DNA strands. There seems to be a preference for nuclear DNA over mitochondrial DNA. (95) These primary lesions block DNA replication and transcription causing cell damage leading to cell death and apoptosis.

Resistance to oxaliplatin is thought to stem from different mechanisms involved in DNA repair. (96) These include nucleotide-excision repair (NER), base excision repair (BER), mismatch repair (MMR) and double strand break repair. This area is currently the focus of much research to help identify possible pathways of resistance and future therapies. (97)

1.10.4 Biological Agents

In order to continue to grow, tumours require the development of their own blood supply. As they enlarge, they reach a point at which they can no longer grow without an independent supply. At a critical point there is relative tissue hypoxia that causes increased expression of transcription factors such as hypoxia inducible factor 1 α (HIF-1 α) which upregulates VEGF and resulting angiogenesis. (98) Expression of EGF, transforming growth factor β (TGF- β) and interleukins 1 and 6 (IL-1, IL-6) have been shown to be involved in the expression of VEGF. (99) Basic understanding of these pathways has led to the development of monoclonal antibodies to directly inhibit them in patients with cancer.

1.10.5 Cetuximab

Cetuximab is a chimeric monoclonal antibody that inhibits the Epidermal Growth Factor Receptor (EGFR). EGFR are found on most colorectal cells and are involved in signaling pathways that are deregulated in cancer cells. Inhibition of the receptor inhibits growth, causes complement activation and mediates antibody dependent cellular cytotoxicity. (100)

Its effect is dependent on the status of the KRAS protein status of the tumour. Patients with KRAS wild type tumours have been shown to benefit from the administration of the targeted antibodies cetuximab and panitumumab.

1.10.6 Bevacizumab

Bevacizumab is a recombinant monoclonal antibody that is a vascular endothelial growth factor inhibitor (VEGF). VEGF is a signaling protein involved in angiogenesis. Expression of VEGF allows tumours to grow and metastasise. (100)

1.11 Chemotherapy-Induced Hepatotoxicity

Despite the potential advantages of neo-adjuvant chemotherapy in colorectal liver metastases, it has to be remembered that there are side-effects, often significant, associated with its use. The increasing number of patients receiving chemotherapy for colorectal liver metastases has some distinct patterns of damage being recognised and associated with the different agents. 5-fluorouracil has been found to be associated with steatosis (101), while irinotecan has been implicated in the development of steatohepatitis. (102) The use of oxaliplatin has been found to lead to development of a vascular condition called sinusoidal obstruction syndrome (S.O.S.) in patients receiving that particular agent. (103)

It is important that a liver that has been damaged by chemotherapy is recognised early as the future remnant liver may not be sufficient to provide adequate function post-operatively. This is especially important when a major resection such as a tri-sectionectomy is planned.

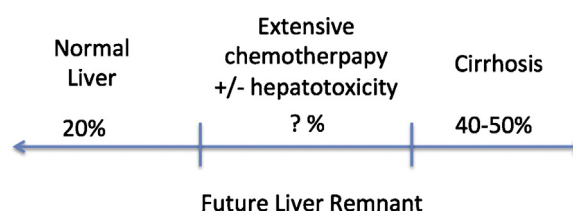


Figure 16: Suggested minimum future liver remnant for patients undergoing surgery. The impact of chemotherapy toxicity is not well defined but caution is advised. (104)

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of conditions ranging from simple steatosis to steatohepatitis, fibrosis and eventually cirrhosis. A summary of the hepatotoxicity associated with chemotherapy is shown in Table 6.

<u>Chemotherapy Agent</u>	<u>Associated Hepatotoxicity</u>	<u>Proposed Mechanism of Hepatotoxicity</u>	<u>Incidence of Hepatotoxicity</u>	<u>Impact of Hepatotoxicity</u>
5-Fluorouracil Capecitabine	Steatosis	Impaired β -oxidation and accumulation of fatty acids.	30-47% (104)	Increased post-operative infections. (105) Hyperbilirubinaemia (106)
Irinotecan	Steatohepatitis	Mitochondrial impairment leading to impaired β -oxidation and inflammation secondary to cytokine release. Potential topoisomerase inhibition of mitochondrial DNA	12-25% (107)	Increased 90-day mortality particularly from liver failure. (107)
Oxaliplatin	Sinusoidal Obstruction Syndrome	Generation of reactive oxygen species (ROS) and glutathione depletion. Upregulation of genes involved in inflammation such as matrix metalloproteinase-9 (MMP-9)	19-78% (103)	Increased peri-operative bleeding and requirement for blood transfusion. (108) Potential reduced long-term survival. (109)
Cetuximab Panitumumab	No recognised hepatotoxicity			
Bevacizumab	No recognised hepatotoxicity			

Table 6: Summary of chemotherapy-induced hepatotoxicity and mechanisms

1.11.1 5-fluorouracil and steatosis

Steatosis is defined as being present when the fat content of the liver is greater than 5% of its wet weight. Its severity is traditionally scored using the Brunt score which is shown below.

Grade	% of hepatocytes affected
Absent	<5%
Mild	5-33%
Moderate	>33-66%
Severe	>66%

Table 7: The Brunt Score for grading of steatosis/steatohepatitis. (110)

Computed tomography has shown that hepatic fat content increases by 30-47% after treatment with 5-FU. (111-113) A meta-analysis has shown that the presence of hepatic steatosis is a risk factor for increased peri-operative morbidity and mortality in patients having major (>3 segment) resections. (114) However several other studies have failed to show any significant increase in complications. (115, 116) It is often difficult to assess the impact of 5-FU in isolation as it is rarely used as a single agent these days. It has also been shown that body mass index (BMI) has a significant correlation with the development of steatosis when receiving chemotherapy. Patients with a BMI >25 had a >20% increased risk ($p=0.02$), while those with a BMI >30 were at increased risk of severe steatosis ($p=0.03$). Other patient factors such as age, gender or diabetes did not affect the risk of developing steatosis. (117)

Although the exact mechanisms of chemotoxicity are still poorly understood, there are some widely accepted hypotheses. As well as breaking 5-FU down to dihydrofluorouracil (DHFU), the rate-limiting enzyme DPD also produces catabolites such as fluoro-beta-alanine (FABL) that are metabolised in hepatocytes. It has been shown that FABL remains in hepatocytes long after cessation of therapy suggesting that the pathways involved are easily saturated. (111) The resulting reduced capacity to metabolise drugs and fat is thought to lead to accumulation of intracellular lipids. 5-FU is also associated with collapse of the mitochondrial membrane leading to impaired oxidation of fatty acids and increased production of reactive oxygen species (ROS) mediated by cytochrome p450 enzymes. The

resulting damage from the ROS and impaired beta-oxidation leads to lipid accumulation and steatosis. (118)

1.11.2 Steatohepatitis and Irinotecan

Steatohepatitis is a more worrying condition. It has been shown to be present in patients with Type 2 diabetes mellitus, obesity and the metabolic syndrome. (119) Vauthey et al found that it was also associated with receiving irinotecan therapy, with 20.2% of patients receiving therapy developing it compared to 4.4% in the chemo-naive group. ($p < 0.001$) (107) The same study also showed that there is a significant increase in surgical morbidity and mortality associated with developing steatohepatitis with an increased 90-day mortality of 14.7% vs 1.6% ($p = 0.001$). However, the data remains inconclusive, as other studies have failed to show any significant difference. (120) Possible confounding factors include differing rest periods after chemotherapy prior to surgery.

The precise mechanism of irinotecan hepatotoxicity is unclear although it is thought to involve a “2-hit” process. The first “hit” is accumulation of fat within the hepatocytes with oxidative stress caused by the chemotherapy providing the second “hit” resulting in the development of the hepatotoxicity. It is thought that mitochondrial dysfunction is at the core of the process. (121) Mitochondrial function and that of the mitochondrial respiratory chain is reliant on the expression of several polypeptides encoded by the mitochondrial DNA (mtDNA) that is located within the mitochondrial matrix. This undergoes continuous replication and constant levels are required for it to function. If the level of mtDNA drops by 20-40% below basal levels, global mitochondrial dysfunction can develop. (118) The dysfunction causes increased production of reactive oxygen species (ROS) through the damaged respiratory chain, increased lipid peroxidation and impairment of beta-oxidation. This can trigger release of pro-apoptotic (TNF- α) and pro-fibrotic (TGF- β) cytokines by Kupffer cells leading

to cell death, inflammation and fibrosis. (121) It is targeting pathways such as these that are the focus of ongoing novel research into new therapies to prevent chemotherapy induced liver injury. It has also been suggested that impairment of mitochondrial topoisomerases and subsequent inhibition of mtDNA replication could be a potential mechanism of irinotecan-induced hepatotoxicity. Pre-clinical work has shown that mitochondrial DNA contains Type 1 topoisomerase in a form similar to nuclear DNA. (122) It has also been shown that this mitochondrial DNA (mtDNA) is sensitive to camptothecin, from which irinotecan was developed. (123) (124) Combined with the knowledge that depleted levels of mtDNA can lead to mitochondrial dysfunction and steatohepatitis, this could explain the mechanism involved in irinotecan/chemotherapy-induced steatohepatitis (CASH)(121)

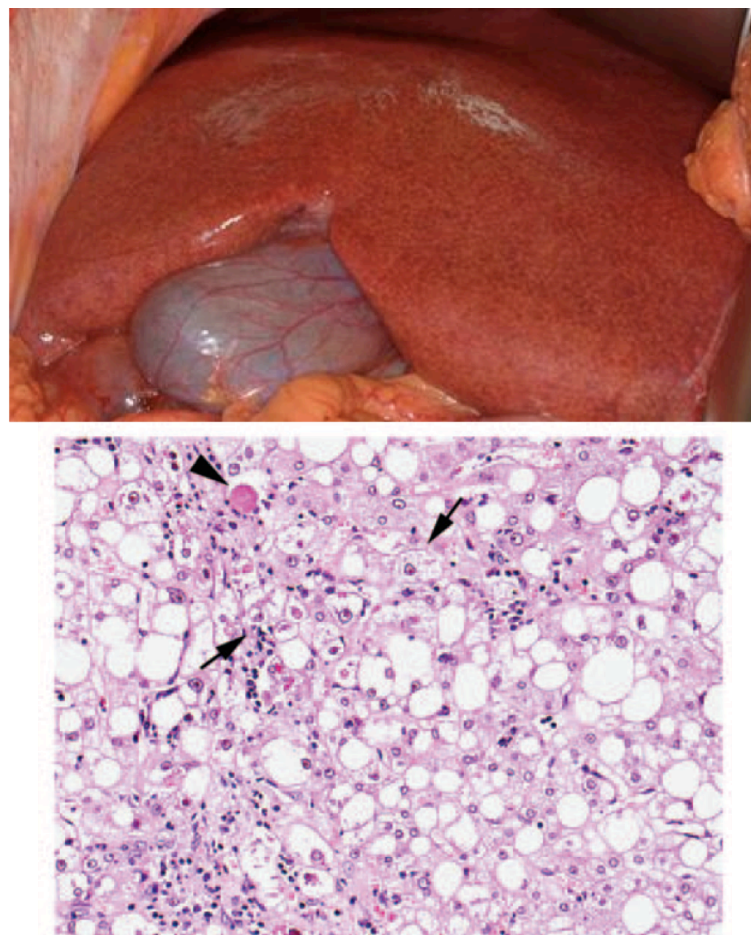


Figure 17: Intra-operative view of a liver showing irinotecan induced steatohepatitis and histology of the same. (Kindly provided by Professor Graeme Poston, Liverpool, UK)

1.11.3 Sinusoidal Obstruction Syndrome and Oxaliplatin

Rubbia-Brandt et al showed that the use of oxaliplatin is associated with the development of sinusoidal obstruction syndrome. (103) They devised a scoring system of increasing hepatotoxicity where 0=absent, 1=mild, 2=moderate and 3= severe. The features of sinusoidal obstruction develop over time starting with sinusoidal dilatation progressing to hepatocyte atrophy, persinusoidal fibrosis and later nodular regenerative hyperplasia.

Work from Vauthey et al at MD Anderson confirmed that there was a significant increase in the incidence of sinusoidal obstruction syndrome in those receiving oxaliplatin therapy than those not (18.9% vs 1.9%, $p < 0.001$) (107) However, a paper from Aloia et al from the same institution, showed that although there were changes in the liver, this did not lead to increased peri-operative morbidity or mortality although there was an increased requirement for peri-operative blood transfusion which itself is a risk factor for complications.

The underlying mechanisms of oxaliplatin induced sinusoidal obstruction syndrome continue to be poorly understood. Ultrastructural abnormalities in the liver after exposure to oxaliplatin has shown that there is an increased rate of endothelial cell apoptosis leading to leaky vessel walls. This leads to extravasation of erythrocytes into Disse's space and deposition of extracellular matrix components including collagen fibres leading to peri-sinusoidal fibrosis. The dilatation of Disse's space and blebs from the endothelial cells bulging into the sinusoidal lumen lead to the obstructive syndrome. (125)

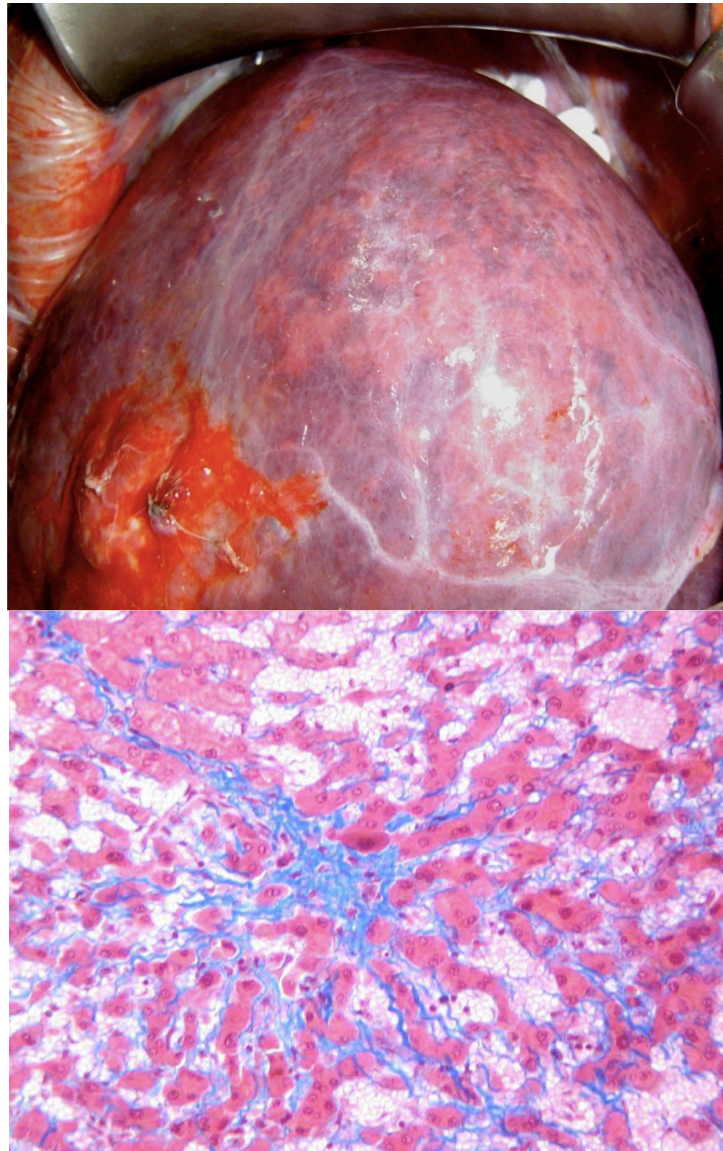


Figure 18: Intra-operative view of the characteristic “blue liver” of oxaliplatin induced sinusoidal obstruction syndrome and histology of the same. (kindly provided by Professor Graeme Poston, Liverpool, UK)

It is thought that increased generation of reactive oxygen species (ROS) and glutathione depletion from sinusoidal endothelial cells causes the increased apoptosis in these cells allowing the damage to occur. Up-regulation and increased activity of matrix metalloproteinase 9 (MMP-9) has also been implicated in the process. (105)

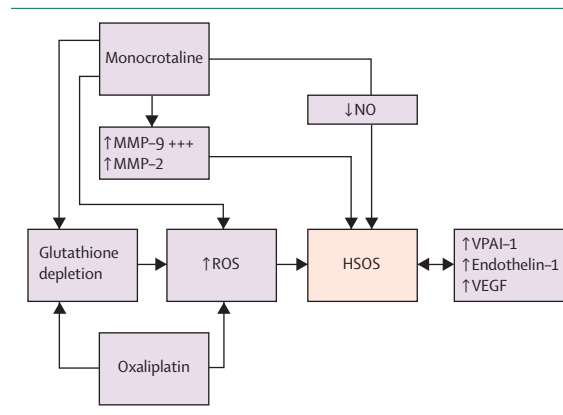


Figure 19: Possible mechanism of the development of Sinusoidal Obstruction Syndrome.

(105)

Recent work has shown that there is up-regulation of several genes in the sinusoidal obstruction syndrome. These included genes involved in regulating angiogenesis, cellular adhesion and inflammation. (126) MMP-9 in particular is associated with pathological processes including cancer invasion, metastasis and angiogenesis. There have been efforts to direct novel therapies into targeting this pathway in the treatment of cancer but work is at an early stage. (127) Of interest, it has been shown that in patients receiving the VEGF inhibitor, bevacizumab, there has been a reduction in the incidence of SOS. Understanding the mechanisms involved in this process could help develop novel ways of preventing it. (125)

1.11.4 Long-term implications of hepatotoxicity

Little is known about the natural history of the conditions caused by chemotherapy, mainly because of small numbers and the deaths of patients from their disease before long-term follow-up is possible. There has been some recent data that in addition to the peri-operative complications, there is a longer-term oncological impact in patients who develop these conditions. Tamandl et al, demonstrated shorter disease free survival ($p=0.05$) and overall survival ($p<0.001$) in patients who had developed sinusoidal obstruction syndrome as a consequence of oxaliplatin. (109)

1.12 Outcomes in Colorectal Liver Metastases

The advances in chemotherapy and surgical technique have had a major impact on the survival in colorectal liver metastases. In a large, retrospective review of over 110,000 patients with colorectal cancer in the United Kingdom between 1998-2004, there were 3116 patients identified who underwent hepatectomy. The overall 5-year survival rate following liver resection was 45.9% (95% C.I. 44.1-47.7%), which was actually better than the 5-year survival rate for those with Stage III disease at 42.2% (95% C.I. 41.7-42.7%). The 5-year survival for non-resected Stage IV was 9.0% (95% C.I. 8.4-9.6%).(128)At present there is no staging system that reflects the fact that Stage IV patients who are resected have as good an outcome as those with earlier Stage III disease. (38)

There is a need for novel biomarkers of chemotherapy-induced hepatotoxicity. Current practice is to monitor patients receiving chemotherapy with standard liver function tests including bilirubin, ALT/AST and alkaline phosphatase. Although the current gold standard, these tests are often not reliable enough to detect hepatotoxicity, as despite normal serum results during chemotherapy, it is not until the time of surgery when the damage is detected, by which time it can have a significant effect on the operative strategy. The need for more sensitive and specific biomarkers is recognised in a number of conditions and the role of serum-based microRNAs have been proposed as a possible solution.

1.13 Micro-RNA

RNA silencing is an evolutionary conserved physiological process regulating gene expression. (129)The first discovery of micro-RNA (miRNA) was lin-4 in *Caenorhabditis elegans* and the subsequent discovery of the miRNA let-7 more than a decade ago. (130; 131) MiRNA are small, non-coding RNAs around 19-22 nucleotides in length. They are involved in post-transcriptional regulation of gene expression in a variety of target genes

involved in key cellular processes such as cell proliferation, differentiation, apoptosis, stem cell development and various human diseases. (132-135)

1.13.1 Biogenesis of miRNA

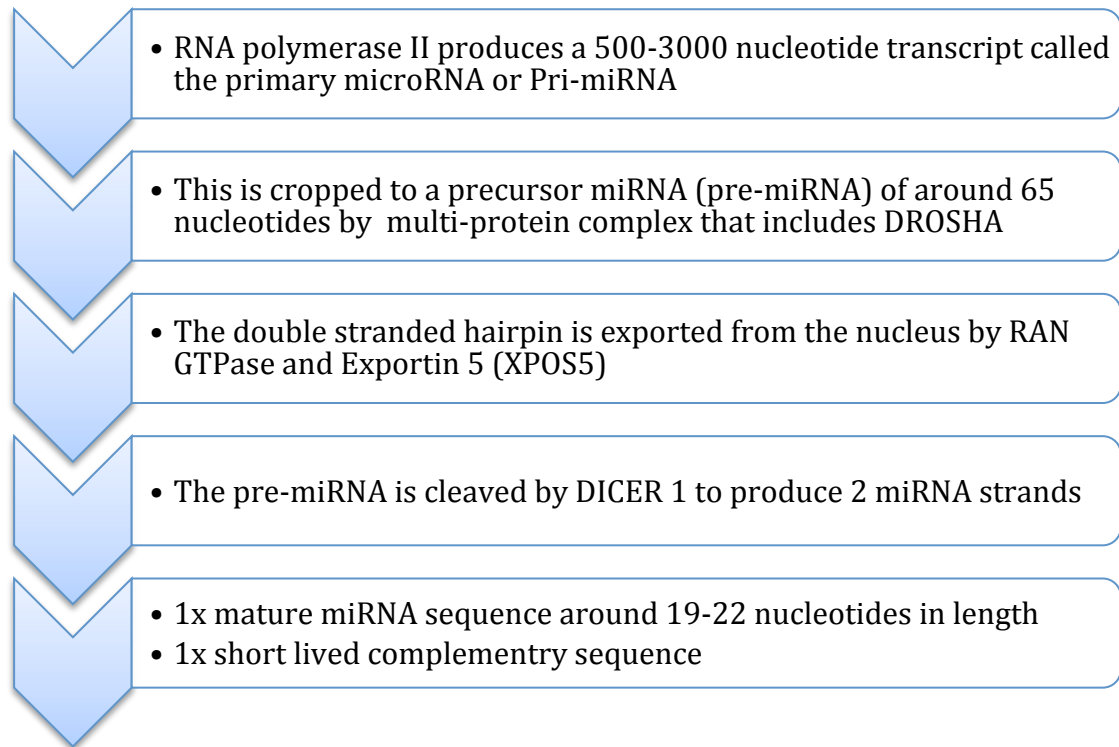


Figure 20: The biogenesis of miRNA. Adapted from several sources. (136-138)

The mature miRNA is then loaded onto the RNA-induced silencing complex (RISC). This recognises and interacts with the conserved complementary target sites in target mRNAs (often in the 3'-UTR) and induces their effect. (139) So far more than 1600 miRNAs have been identified in humans and it thought that more than a third of human protein coding genes are modulated and controlled by miRNAs. (140) MiRNA profiling studies have shown that they exhibit function limited to specific cell types, diseases or stages of development despite the fact that some of them are widely expressed. (141)

1.14 MicroRNA 122

MicroRNA-122 is a liver specific miRNA expressed almost exclusively in the hepatocyte with between 50,000 and 135,000 copies per human cell. (142) MiR-122 accounts for around 70% of all liver miRNA.

1.14.1 Role of miR-122 in liver development

It has been shown that there is a significant spike in levels and up-regulation of miR-122 during embryonic development of the liver. This has suggested a role for miR-122 in the regulation of hepatocyte differentiation, liver development and maintaining the adult phenotype. (143) In a study in mice, Xu et al identified four liver specific transcription factors namely hepatocyte nuclear factor (HNF)-1 α , 3 β , 4 α and CCAAT/enhancer-binding protein α (C/EBP- α), that activated miR-122 expression. This caused a down regulation of genes involved in the differentiation of hepatocytes. (144)

1.14.2 Role of miR-122 in lipid metabolism

MiR-122 was the first miRNA to be identified as having a role in regulating lipid metabolism. (142) Because it is plentiful and specific to the liver it has been hypothesised that it has a role in cholesterol and fatty acid metabolism. There have been a number of studies using mouse models where antisense strategies have been used to sequester miR-122. The mice have been found to decrease levels of cholesterol, low density lipoprotein (LDL) and high density lipoprotein (HDL) in both the liver and bloodstream. There was also decreased fat accumulation in the liver. (145-148) In mice fed with high fat diets, miR-122 silencing has led to decreased hepatic steatosis, reduced cholesterol synthesis rates and increased hepatic fatty acid oxidation. This has led to interest in a potential role for miR-122 in the treatment of dyslipidaemias.

1.14.3 miR-122 and Hepatitis C Virus

Hepatitis C (HCV) is a positive sense RNA-virus affecting around 180 million people worldwide. It is associated with chronic liver disease and the development of fulminant hepatic failure. Jopling et al were the first to identify the important role of miR-122 in the replication and development of HCV. They found that there were two miR-122 binding sites on the viral mRNA. One was found to be in the 5'-non-coding region (NCR) and the other in the 3'-NCR. It was shown that miR-122 needed to bind to the viral mRNA for replication to occur. Treatment to sequester and inhibit miR-122 led to a significant decrease in replicating viral mRNA. (149) Since then a number of other studies have been performed with similar outcomes adding weight to the hypothesis that miR-122 is essential for HCV development. (150-153) There is also evidence that the miR-122/RISC complex actually stabilises the HCV viral RNA conferring protection against damage to it. (154) There is currently a lot of interest in the potential use for miR-122 based therapies in the treatment of HCV.

1.14.4 miR-122 and Hepatitis B Virus

Hepatitis B Virus (HBV) is a DNA virus affecting around 350 million people worldwide. It causes acute and chronic infection that can lead to cirrhosis and hepatocellular carcinoma (HCC). The role of miR-122 and HBV has been less extensively investigated than its sister virus, HCV. Despite this, it has been shown that miR-122 also plays an important role in the regulation of HBV. In animal models it has been shown that transfection of miR-122 into a cell inhibits HBV replication while antisense inhibition of miR-122 causes an increase in HBV replication. This pattern is the complete opposite in contrast to its role in HCV. (155) Further studies have confirmed that miR-122 strongly inhibits HBV expression and replication as well as acting to suppress cell proliferation and malignant transformation of hepatocytes. (156-158) These findings raised the question of why HBV existed in such a

place where miR-122, a potent inhibitor, is so highly expressed. Wang et al attempted to answer this and found that in patients with HBV, intracellular expression of miR-122 in hepatocytes was significantly reduced. (159) This has led to exciting hypotheses that the HBV virus interacts with its host via unknown pathways to cause down-regulation of miR-122 to allow its survival.

1.14.5 miR-122 and Hepatocellular Carcinoma

There is increasing evidence and interest in the role of miR-122 in carcinogenesis, particularly hepatocellular carcinoma (HCC). MiR-122 expression has been shown to be reduced in these tumours, and the degree of down-regulation has been shown to be associated with prognosis. Lower expression of miR-122 is associated with poor prognosis and metastases. (160-163) MiR-122 has been shown to be involved in cell proliferation, apoptosis, migration, invasion and tumour formation. Inhibition of miR-122 increases the rate of these functions while restoring the miR-122 reverses the effect. Over-expression of miR-122 has been shown to sensitise the tumour to the effects of chemotherapy. (164-166) There is intense interest in the use of miR-122 in the treatment of HCC, particularly as currently it is associated with such a poor prognosis.

1.15 miR-122 as a biomarker

Novel biomarkers are essential to facilitate pre-clinical and clinical research and practice. In the field of drug-induced liver injury (DILI) there is an urgent need for new, more sensitive and specific biomarkers. The official Federal Drug Administration (FDA) definition of a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathological processes or pharmacological responses to a therapeutic intervention”. In practice a biomarker must be

readily available in as non-invasive manner as possible. It must be specific and sensitive and the test must be easily reproducible. Sensitivity measures the proportion of actual positives which are correctly identified as such (true positive rate). Specificity measures the proportion of negatives which are correctly identified as such (true negative rate). It has been shown that miRNA is highly stable in plasma. Coupled with its relative abundance and tissue specificity, it has been proposed as a potential biomarker in a number of situations. (167) With its high specificity for liver, there is increasing interest in the potential use of miR-122 as a biomarker for DILI.

Current biomarkers of hepatotoxicity are bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) known, incorrectly as liver function tests (LFTs). In particular the enzymes ALT and AST are used as markers of hepatocyte damage. Severe hepatotoxicity is defined as levels >3 times the upper limit of normal (ULN). During the necrosis of hepatocytes the enzymes leak into the systemic circulation. Serum ALT level has been shown to be correlated with the amount of hepatocyte damage. (168) The drawback with ALT and AST is that they are not solely found in liver. Two isoforms of ALT exist, ALT 1 and ALT2. These are also expressed in lower but significant levels in other tissues such as muscle, bowel and myocardium. Levels can be raised above normal in conditions such as myositis and myocardial infarction. (168) In fact ALT and AST have previously been used as “cardiac enzymes” as biomarkers of acute myocardial infarction prior to the discovery of cardiac-specific troponins.

Wang et al produced a landmark paper on the use of miR-122 in DILI. Using mice they showed that after receiving a toxic dose of paracetamol, miR-122 levels increased earlier than ALT and at lower concentrations of paracetamol suggesting that it is more sensitive than the current markers. (169) Yamaura et al published similar findings in rats confirming its potential as a biomarker across species. (170) Following on from this there have been studies in a human population. In our own group, Starkey-Lewis et al confirmed its potential as a biomarker in patients with acute liver failure following paracetamol overdose. Their findings were similar to those

of Wang et al, showing that there is good correlation between miR-122 and ALT, but that the rise in miR-122 occurred at an earlier time point. (171)

The role of miR-122 as a biomarker in a variety of conditions has been explored. Different aetiologies of hepatotoxicity in which miR-122 has been suggested as a potential biomarker include HCV (172), HBV (173-175), HCC (173; 174), acute liver transplant rejection (176) and alcoholic liver cirrhosis (177) Yang et al explored the use of biofluids other than blood. Having given rats toxic doses of paracetamol and other drugs, they were able to measure raised levels of miR-122 in urine. (178) There is currently major interest in the potential uses for miR-122 as a biomarker in a range of liver injuries.

Aims and Hypotheses

Over the course of this thesis the aim is to assess the potential role for microRNA-122 as a serum biomarker for the early detection and diagnosis of chemotherapy-induced hepatotoxicity in patients receiving neo-adjuvant chemotherapy for advanced colorectal cancer with liver metastases. In particular the following hypotheses will be tested.

- That miR-122 is detectable in the serum of healthy human subjects.
- That miR-122 exists within a narrow range in the normal population and does not vary with time.
- Patients with liver disease/injury will have higher levels of miR-122 than a normal population.
- MiR-122 will be more sensitive than serum transaminases in liver disease and injury.
- MiR-122 is of use in diagnosing hepatocellular carcinoma (HCC) within an at-risk patient group.
- That miR-122 can detect chemotherapy induced liver injury in patients undergoing neo-adjuvant chemotherapy for colorectal cancer liver metastases.

- That miR-122 is more sensitive and specific than transaminases at predicting chemotherapy induced liver injury.

Chapter 2

Defining the behaviour of miR-122 in a healthy
population

2.1 Introduction

Chemotherapy-induced hepatotoxicity is a common problem for patients undergoing treatment for colorectal liver metastases affecting up to 78% of all patients. (179) Patients receiving chemotherapy are monitored both clinically and biochemically during their treatment to detect side-effects that may develop. Unfortunately, despite this monitoring, patients continue to develop significant liver injury that is not detected until laparotomy when it may impact on outcome. (105; 107-109)

To date, only a relatively small number of tests are used to assess liver integrity. These primarily consist of serum total bilirubin (marker of hepatic function) and the determination of the serum activity of the enzymes alkaline phosphatase (marker of biliary injury) and aspartate aminotransferase (AST) and alanine aminotransferase (ALT), markers of hepatocellular injury.

Serum ALT activity has become the primary screening tool for the detection of liver injury and disease. However, changes in ALT activity occur in a number of liver disease etiologies such as drug-induced liver injury (DILI), viral hepatitis, fatty liver disease and liver cancer. Expression of ALT is not specific to the liver and increased serum levels can also be detected in other conditions such as myocardial damage, muscle toxicity, myositis and extreme exercise. Furthermore, ALT activity does not always correlate with histopathological damage that results in a significant impediment to the interpretation of its results. (168) Recently, the use of the hepatocyte-specific microRNA-122 (miR-122) has been proposed as a superior biomarker for hepatic injury and disease.

Proposed potential advantages of miR-122 over conventional tests are increased organ specificity, presence in a non-invasive biofluid (blood, urine) and low baseline variation in the healthy population. Furthermore, the development and validation of assay methodologies that are robust, translational and can be used in many laboratories is critical to the

development and qualification of novel experimental biomarkers such as miR-122. Therefore, the objective of this investigation was to quantify the circulating level of miR-122 in a healthy population and to define a normal reference range, degree of variation and associate potential impact of age and sex that can be used to assess changes found in patients with hepatic injury and disease.

2.2 Patients and Methods

129 healthy volunteers were recruited as part of the BIOPAR study run by the University of Liverpool. These volunteers were recruited as a control group for the study. All had no significant past medical history, were non-smokers and were on no medications. Peripheral blood was taken from these volunteers and serum/plasma was obtained by centrifugation and stored at -80°C until analysis. MicroRNA-122 levels were obtained by reverse transcription real-time polymerase chain reaction (qRT-PCR) along with two endogenous controls and one exogenous control spiked in to the sample to act as a technical control. The endogenous controls were the small RNA, U6snRNA, found in the nucleus of most cells and the endogenous miRNA, let-7d. These molecules were chosen as they had been described in the published literature and had been used as part of a validated assay within our own research group. For the exogenous control we used C.lin-4, a non-mammalian microRNA.

For 50 patients described above, serial samples taken over 3 days were analysed to investigate natural variation within each patient.

A further 24 patients had samples taken at 9 different time points over a 24-hour period to investigate circadian variation within each patient.

2.2.1 miRNA extraction

Starkey-Lewis et al have previously described the method used for analysis of the miR-122 used by our group in detail in 2011. (171) In brief, miRNA was extracted using an miRNeasy kit (Qiagen, Venlo, Netherlands) following the manufacturer's instructions with a few minor modifications. Briefly, 40uL of biofluid was made up to 200μL with nuclease free water then combined with 700μL of QIAzol. The sample was mixed and left to incubate for 10 minutes at room temperature before the addition of 140μL of molecular grade chloroform. At this point 5uL of C.lin-4 was spiked in. The samples were vortexed for 15 seconds and centrifuged at 12,000g for 15 minutes at 4°C. Equal volumes (350μL) of the aqueous upper phase and 70% ethanol were mixed in a fresh microtube before adding the total volume to an miRNeasy minispin column. The column was centrifuged at 8,000g for 15 seconds at room temperature. The flowthrough, containing the small RNA fraction (including the miRNA) was mixed with 450μL of 100% ethanol. The elute was then purified using an RNeasy MinElute kit (Qiagen, Venlo, Netherlands).

2.2.2 miRNA purification

The small RNA elute was applied to the RNeasy MinElute column 700μL at a time and centrifuged at 8,000g for 15 seconds at room temperature. The miRNA immobilised in the columns was then washed with various buffers before a final 80% ethanol wash. The column was then air-dried by centrifuging with the lid open. The miRNA fraction was then eluted in 14μL of nuclease free water.

2.2.3 Reverse Transcription to cDNA

The miRNA elute was reverse transcribed using specific stem-loop reverse transcription RT primers (Applied Biosystems, Foster City, CA) for each target miRNA species following the manufacturer's instructions. 2μL of RNA was used to produce the complementary DNA (cDNA) template in a total volume of 15μL.

2.2.4 Quantitative Polymerase Chain Reaction Analysis

1.33 μ L of cDNA was used in the PCR mixture with specific stem-loop PCR primers (Applied Biosystems, Foster City, CA) in a total volume of 20 μ L. Levels of miRNA were measured by the fluorescent signal from the Taqman probes on an ABI Prism 7000 (Applied Biosystems). All samples were assayed in duplicate. Results were expressed as the cycle threshold (Ct), defined as the PCR cycle number at which the signal rises above the baseline. When performed, normalisation was done using the Δ Ct method (Ct target RNA- Ct control RNA).

2.2.5 Relative Quantification using a Standard Curve

A standard curve was defined using a synthetic oligonucleotide of miR-122 (IDT, Leuven, Belgium). A serial dilution from a starting concentration of 10^9 copies per PCR well was made down to 10^2 copies per well. This was assayed in duplicate with three replicates. Applying PCR CT values to this standard curve made relative quantification.

2.2.6 ALT Levels Determination

Serum ALT levels were determined in the hospital laboratory of the Royal Liverpool University Hospital after sampling.

2.2.7 Statistical Analysis

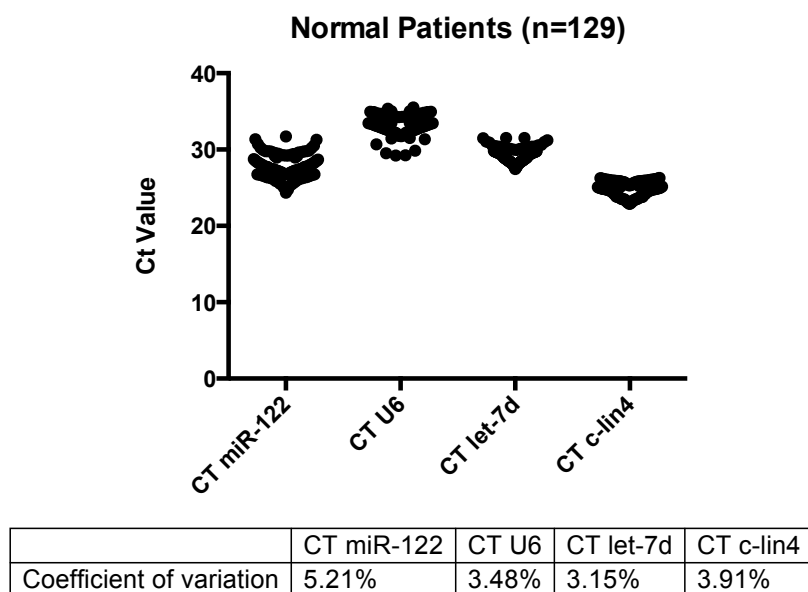
Descriptive statistical analysis was performed on the patients including medians and range. To determine statistical significance, comparisons were made using the Students t-test (for parametric data) and the Mann-Whitney U test (for non-parametric data). Correlative analysis was made using Pearson's test and variation was assessed using co-efficient of variation which is a ratio of the standard deviation to the mean, showing the extent of

variability in relation to the mean of the population. All statistical analysis was carried out using GraphPad Prism software (GraphPad Software, La Jolla, CA). Statistical significance was set at $p=0.05$. Normal reference ranges were calculated as the 95% prediction interval of the population.

2.3 Results

129 healthy volunteers were included for analysis in this study. The median age was 35 with a range of 18-66. The male to female ratio was 1:1.4.

We measured serum miR-122 as well as two endogenous controls (U6snRNA, let-7d) and one exogenous control (C.lin-4). Mean Ct value for miR-122 was 27.79 (95% C.I. 27.54-28.05), U6snRNA 33.19 (95% C.I. 32.99-33.39), let-7d 29.27 (95% C.I. 29.10-29.45) and C.lin-4 25.00 (95% C.I. 24.83-25.17). Co-efficient of variation for miR-122 was 5.21%, U6snRNA 3.48%, let-7d 3.15% and C.lin-4 3.9%. (Figures 21-22)



	Δ Ct miR-122/U6	Δ Ct miR-122/let-7d	miR-122 copy number/uL
Coefficient of variation	106.37%	78.53%	84.67%

Figure 21: Ct values of miR-122 and controls. Depending on the method of expressing the data used, there is a wide variation in the %CV.

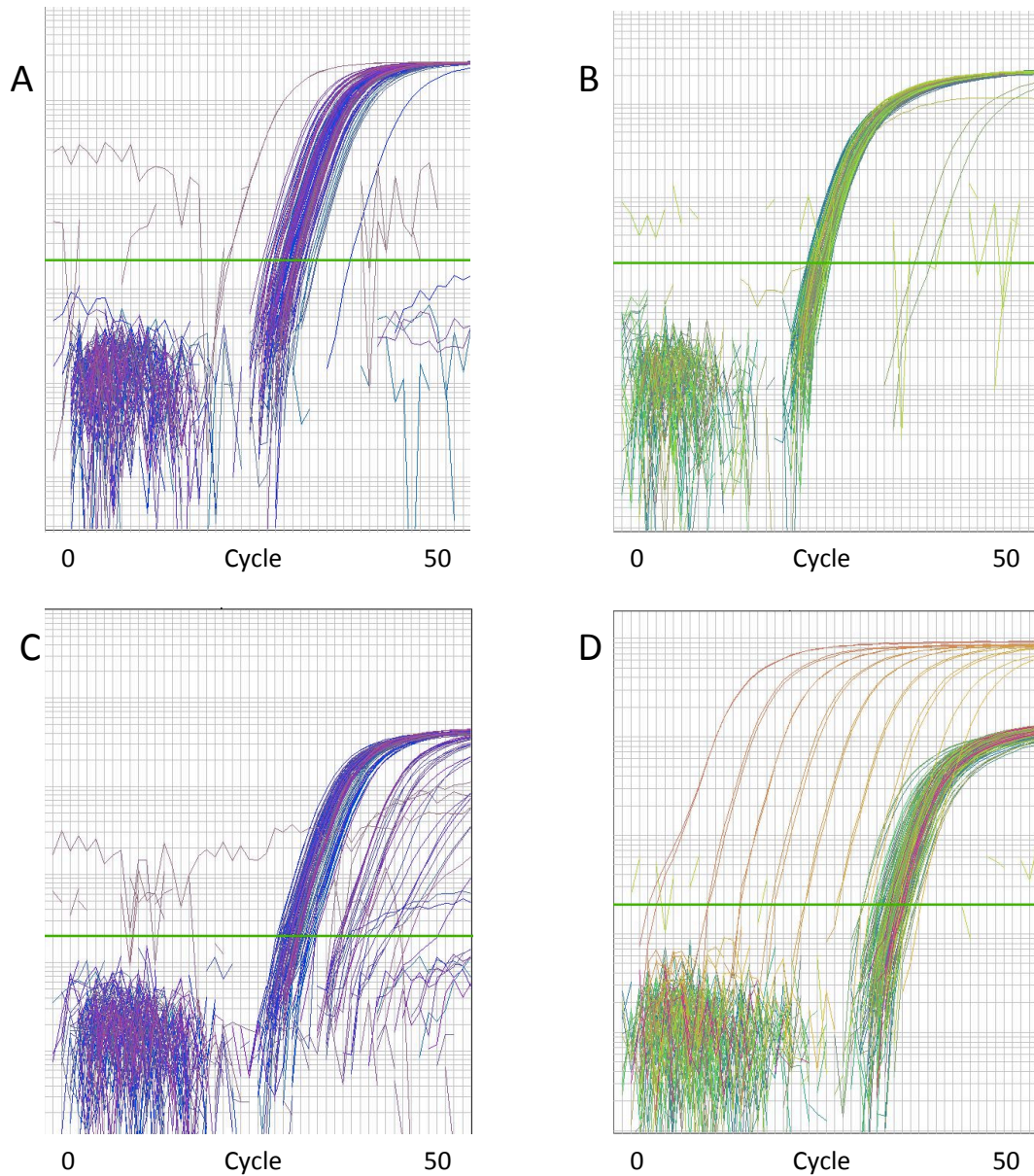


Figure 22: PCR amplification plots of miR-122 (A), c-lin4 (B), let-7d(C) and U6snRNA (D). All showed low %CV; miR-122 5.2%; c-lin4 3.91%; let-7d 3.15%; and U6snRNA 3.48%.

Median age was 35 years. No significant difference was seen when compared between the groups 18-35 years old and the group 35-66 years old. Mean Ct values for miR-122 were 27.83 (95% CI 27.46-28.19) and 27.76 (95% CI 27.39-28.12), $p=0.8$ respectively. The same was seen for the controls: U6snRNA 33.14 (95% CI 32.86-33.42) and 33.23 (95% CI 32.94-33.53), $p=0.65$; and let-7d 29.11 (95% CI 28.84-29.38) and 29.41 (95% CI 29.18-29.64), $p=0.08$. (Figure 23)

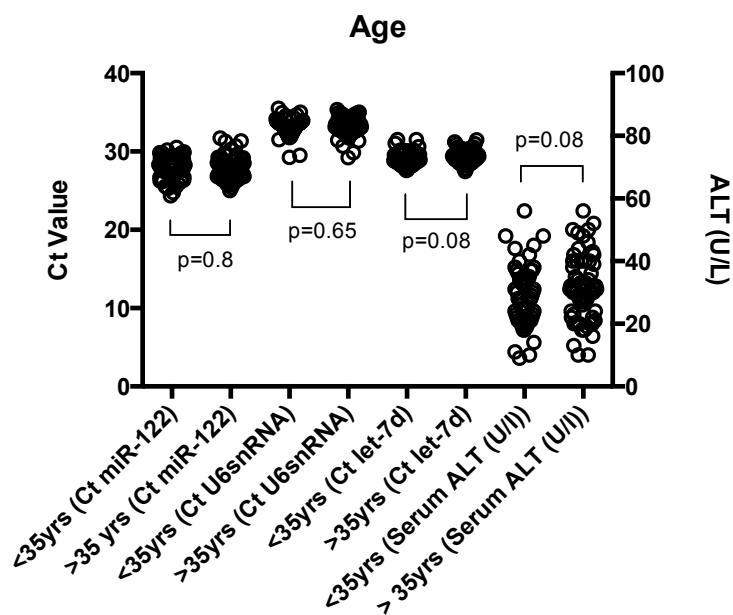


Figure 23: Ct values by age showing no significant differences for any of the targets.

Ct values were also compared between genders. No significant difference was seen in either miR-122 or any of the controls. Mean Ct value for miR-122 was 27.79 (95% CI 27.45-28.13) for females and 27.80 (95% CI 27.40-28.20) for males, $p=0.96$. U6snRNA was 33.17 (95% CI 32.89-33.46) for females and 33.21 (95% CI 32.93-33.50) for males, $p=0.85$. Let-7d was 29.21 (95% CI 28.97-29.44) for females and 29.37 (95% CI 29.09-29.65) for males, $p=0.36$. (Figure 24)

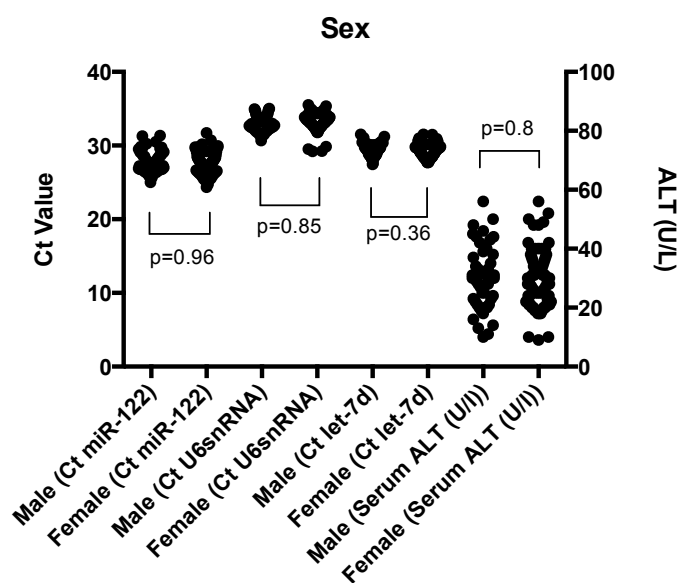


Figure 24: Ct values by gender showing no significant difference.

In order to correct for biological variation and technical variation normalisation with the two commonly used control RNA's was compared. Normalising to U6snRNA produced a greater range of results than the let-7d. Median value for U6snRNA was 51.45 (interquartile range 17.27-96.17) while for let-7d median value was 3.824 (IQ range 1.865-7.621). (Figure 25).

Despite the apparent wider range with U6snRNA, correlation between the two normalisers was good with Pearson's $r=0.67$, $p<0.001$. (Figure 26).

The exogenous control (c.lin-4) showed significant degradation over time. The same amount was spiked-in to each sample and the low variation confirms the technical quality of the extraction process although the variable Ct value poses problems for its use in normalization of data. (Figure 27)

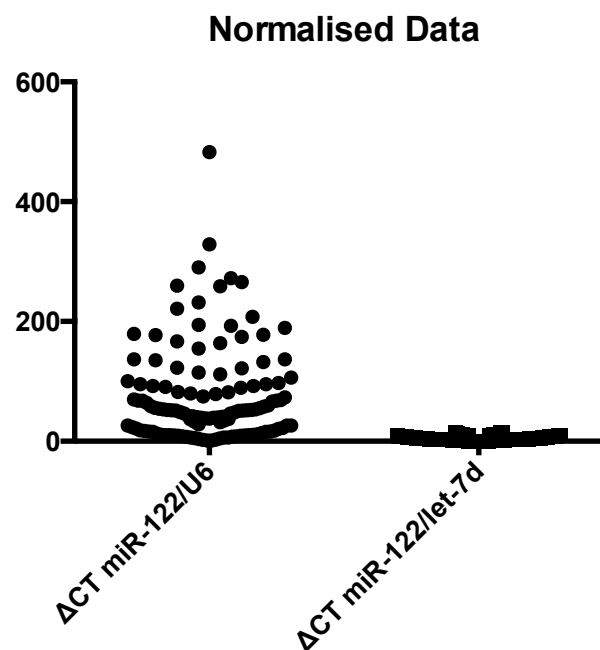


Figure 25: miR-122 normalised to U6snRNA and let-7d. A significant difference is seen in %CV depending on the normaliser used (106.37% vs 78.53%).

Correlation between methods of normalisation

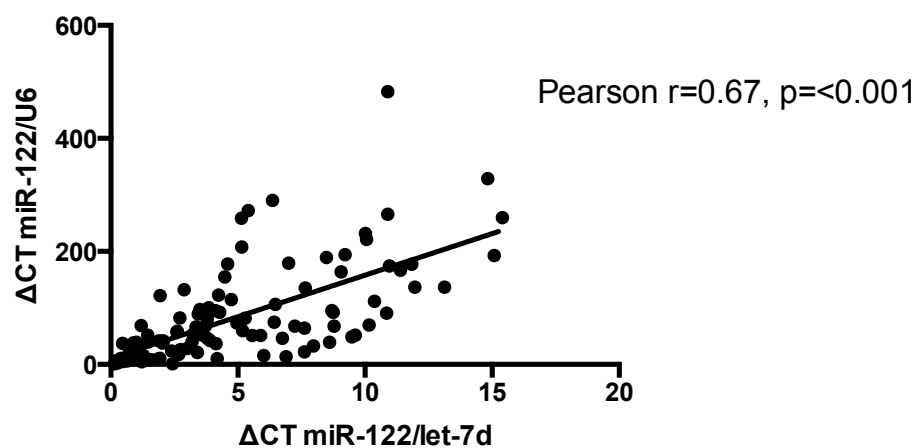
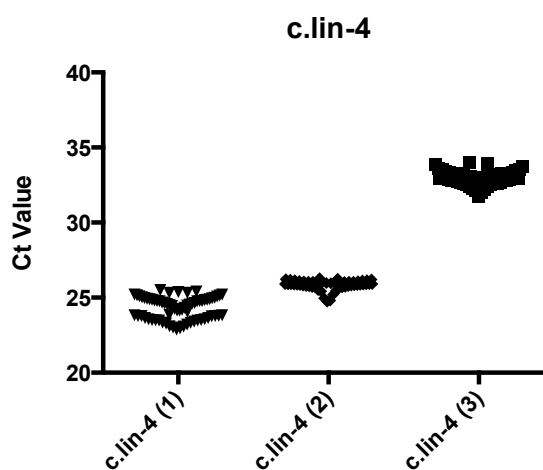


Figure 26: Correlation between both methods of normalisation. Despite the different %CV by method of normalisation, the results correlate well with each other.



	c.lin-4 (3)	c.lin-4 (1)	c.lin-4 (2)
Coefficient of variation	1.43%	3.04%	1.22%

Figure 27: Ct values of synthetic spiked-in miRNA over 3 different experiments. (1) Fresh stock used; (2) Fresh stock used 1 week later; (3) Sample (2) having been stored in -80 degree freezer over a weekend. The potential for degradation of the sample may limit the use of exogenous controls as a method of normalisation.

A standard curve was produced using the synthetic miR-122 oligonucleotide. The R^2 was 0.987 after three replicates run in duplicate. (Figure 28-29)

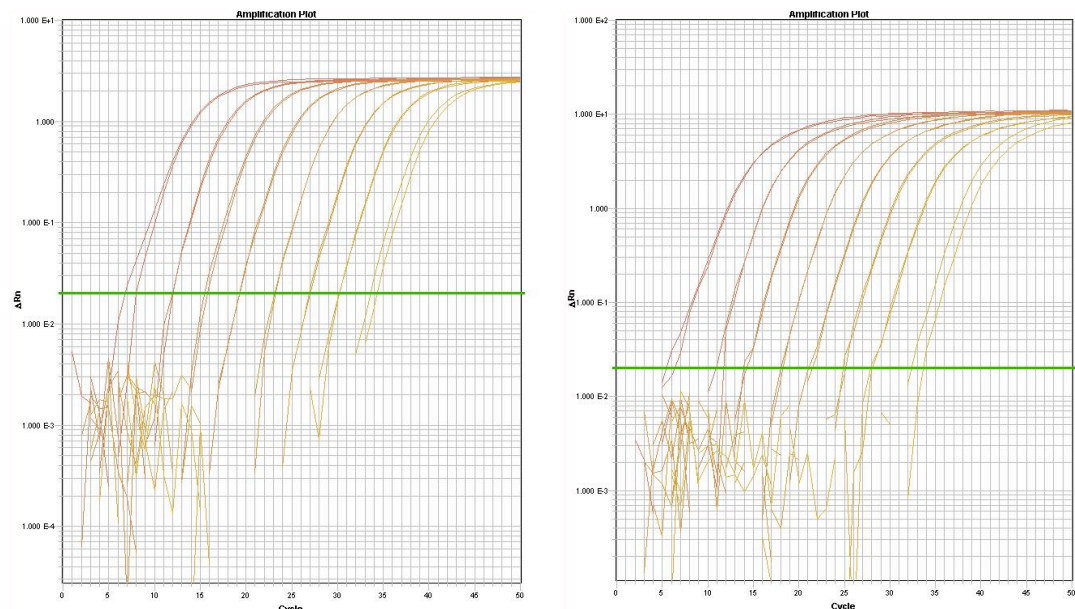


Figure 28: PCR amplification plots of 2 standard curves.

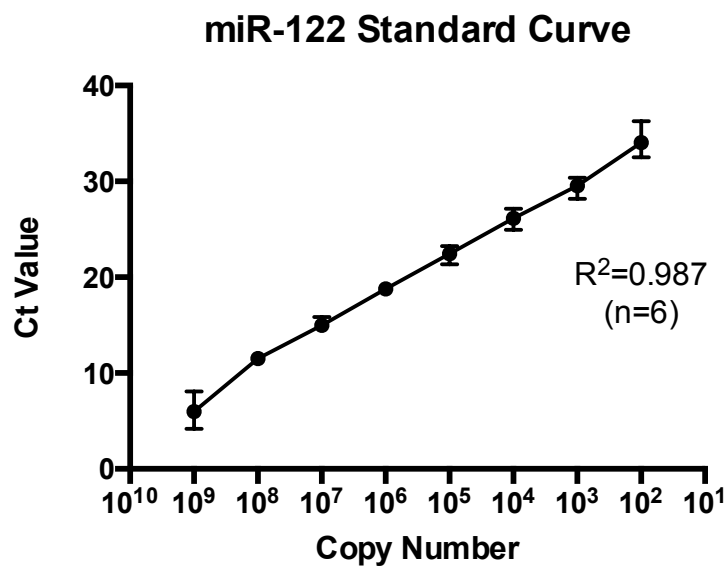


Figure 29: Standard Curve using miR-122 synthetic oligonucleotide with excellent coefficient of determination (0.987). The equation of this line is used to calculate the number of miR-122 copies/uL of serum.

Using the standard curve for quantification, we calculated the number of copies of miR-122 per microlitre of serum. The median value was 12088 copies (IQ range 5256-18347). (Figure 30)

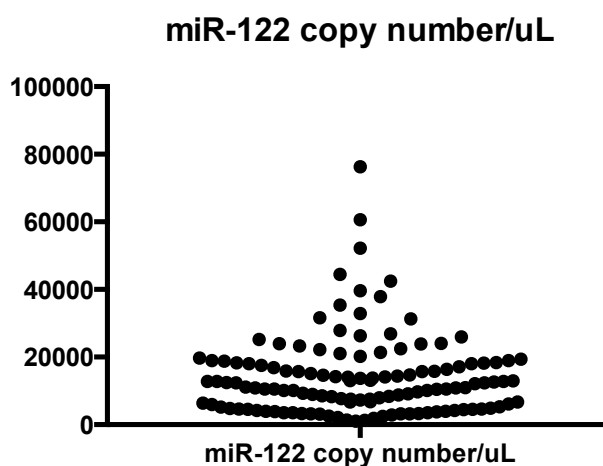


Figure 30: miR-122 copy number per uL of serum.

Serum ALT for all patients was within the accepted normal reference range (<50 IU/L) except one where the result was 52 IU/L.

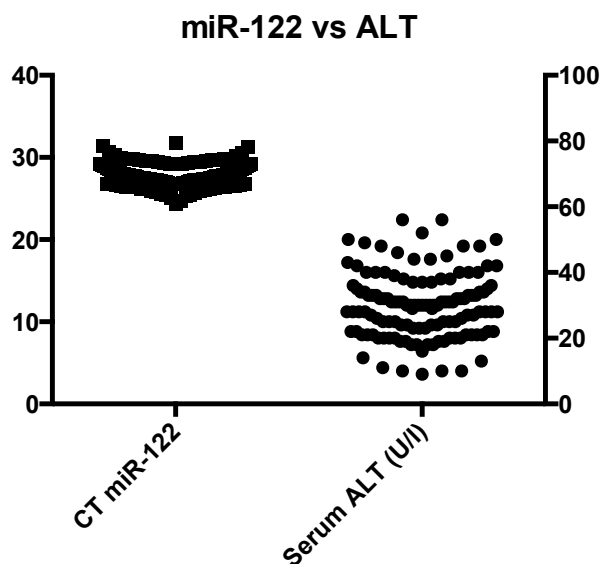


Figure 31: miR-122 and ALT. All but one volunteer had a normal serum ALT. The abnormal ALT was barely raised at 52IU/L (ULN 50).

Reference ranges for mir-122 copy number/uL of serum as well as ΔCt miR-122/U6snRNA and ΔCt miR-122/let-7d were calculated. The upper and lower limits of the reference range were defined 95% prediction interval of the population. (Table 8-9).

Biomarker	Mean	Median	Lower reference range	Upper reference range
ALT	29.78	30.00	13.50	48.50
miR-122 (CT)	27.79	27.52	25.51	30.23
miR-122 (CT/U6) ¹	42.10	51.42	4.81	259.82
miR-122 (CT/let-7d) ²	4.08	3.84	0.37	11.90
MiR-122 (copynumber) ¹	10301	12088	2489.91	38948.67

Table 8: General Reference Ranges for miR-122 expressed in a number of different ways.

Biomarker	Gender	Mean	Median	Lower reference range	Upper reference range
ALT	Female	29.59	28.00	16.40	49.20
	Male	30.04	30.00	12.50	48.50
miR-122 (CT)	Female	27.79	27.75	25.34	30.03
	Male	27.80	27.43	25.82	30.79
miR-122 (CT/U6) ¹	Female	40.85	46.06	5.10	262.43
	Male	43.38	57.97	3.32	225.88
miR-122 (CT/let-7d) ²	Female	3.88	3.42	0.40	13.99
	Male	4.32	4.24	0.28	11.90
MiR-122 (copynumber) ¹	Female	10404	10614	2807.36	43044.94
	Male	10310	12708	1790.05	32532.67

Table 9: Reference ranges by gender

Analysis of the intra-patient variation over 3 days showed little variation (Figure 32) with the mean standard deviation and 95% confidence interval being shown in Table 10.

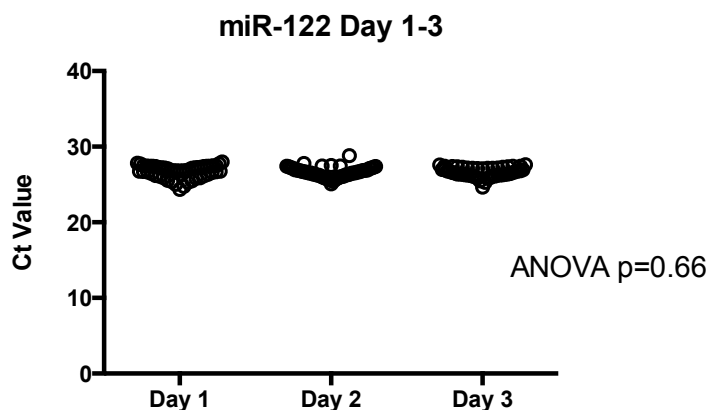


Figure 32: 3 day individual variation of miR-122 by Ct value in 50 healthy volunteers. There was no significant difference over this time period. ($p=0.66$).

Biomarker	N	Mean Standard deviation	95% Confidence interval
ALT (U/L)	50 x 3	1.54	(1.44, 1.64)
MIR-122 (CT)	50 x 3	1.019	(1.016, 1.022)
MIR-122(DCT/U6)	41 x 3	2.66	(2.29, 3.09)
MIR-122(DCT/let-7d)	50 x 3	2.13	(1.86, 2.44)
MIR-122(COPY number/UL)	50 x 3	1.33	(1.27, 1.40)

Table 10: Variation of miR-122 over 3 days in 50 healthy volunteers.

Circadian variation was assessed for each of the target RNAs in 24 patients over 24 hours. No significant variation was seen over 24 hours as shown in Figure 33.

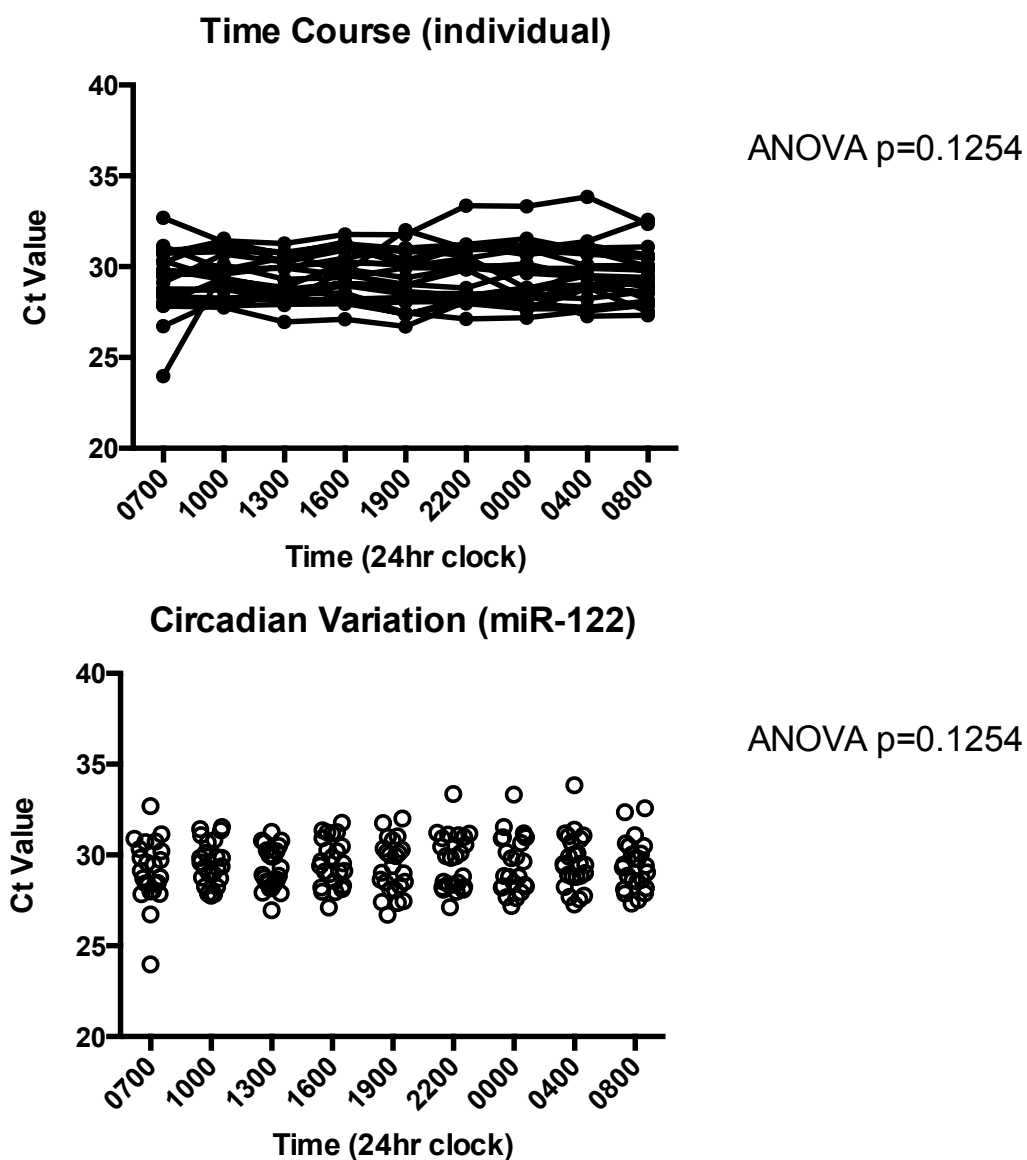


Figure 33. Circadian variation of miR-122 and control RNAs in 24 healthy volunteers.

Assessment of individual patients showed that there was low intra-patient variance over the 24hr period. The median Coefficient of Variation and range (intra-patient) for miR-122 was 1.81% (0.7-3.36%), let-7d 2.33%

(1.35-7.42%), U6snRNA 3.04% (1.48-5.91%) and c.lin-4 0.7% (0.38-3.77%). (Figure 34)

The CV% for inter-patient variation is shown in Figure 35 and is in keeping with the results from the previous normal patients.

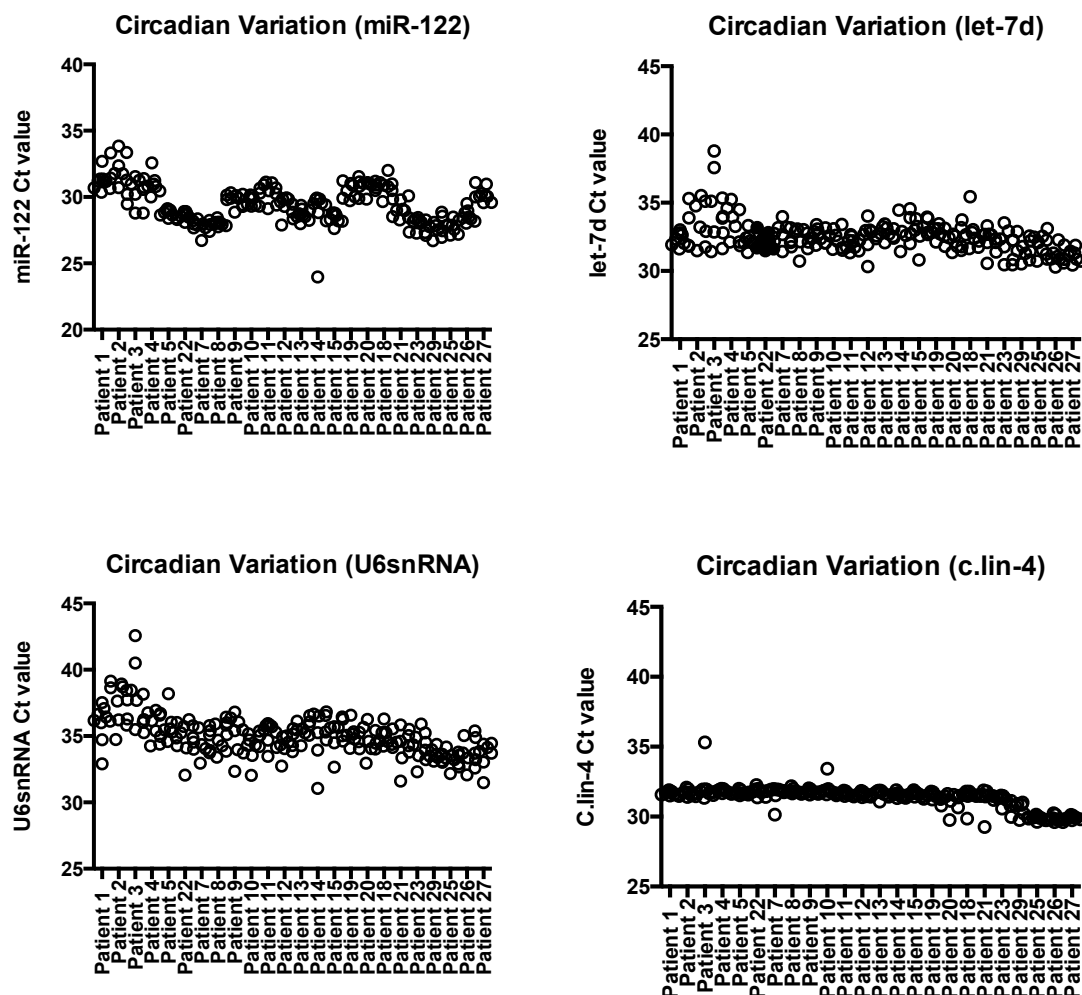


Figure 34. Circadian variation for each individual patient.

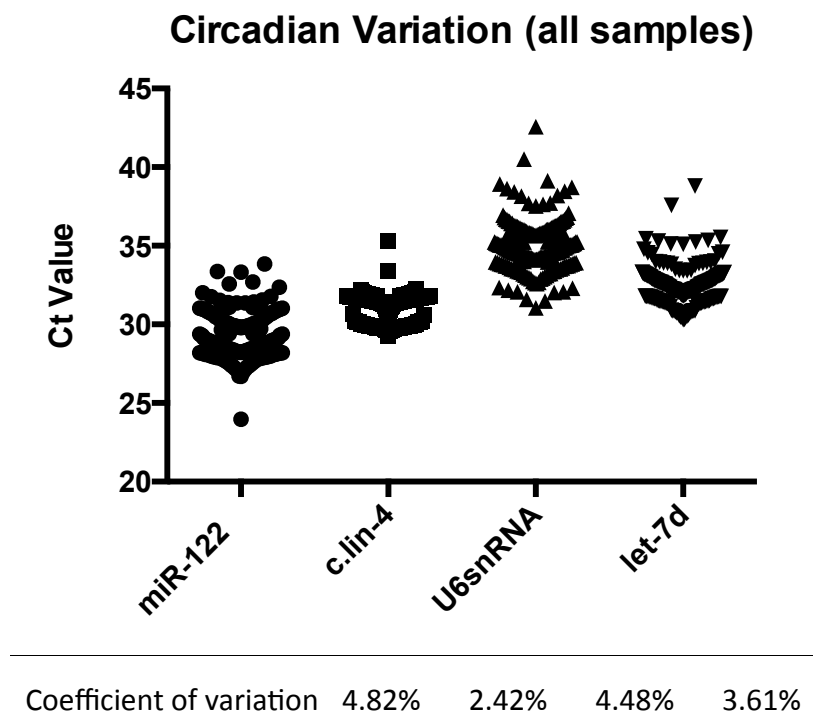


Figure 35. Variation across all 24 patients with CV% in keeping with the results of the other normal patients (n=129)

2.4 Discussion

To date, within the published literature, miR-122 has become increasingly recognised as a promising, potential biomarker for liver injury and disease. However, robust definitions of the degree of intra and inter-individual variation and the impact of age, sex and diurnal effects have not been established for circulating miR-122 within the healthy population. Establishing these parameters in healthy individuals is essential for the interpretation of miR-122 data measured in disease cohorts and for understanding its 'fit for purpose' as a biomarker of liver injury and disease. The objective of the current investigation was to define reference range values for circulating miR-122, and commonly used normalizing RNA controls, by reproducible and robust PCR methodologies in a healthy human cohort. Furthermore, we sought to investigate the impact of age and gender

on intra and inter-individual variation of circulating miR-122 in healthy individuals.

The data obtained from this investigation show that miR-122 is detectable in the serum of healthy patients and that it has a very low degree of inter-individual variation. Furthermore, we have also shown that there was no significant variation when age or gender was taken into consideration. These findings were also reproduced for both of the endogenous controls commonly used for normalization of circulating miR-122.

The study also showed that there is little temporal variation, both in terms of a circadian variation over 24 hours and over 3 consecutive days. Of interest is the difference between intra- and inter-patient variation. The CV% of the population is excellent at around 5%, but the 24 hour sampling revealed that the intra-patient variation is even less, at under 2%. This could be beneficial if using serum miR-122 as a long-term marker in the same patient to assess response to therapy or development of hepatotoxicity, as is done in Chapter 4.

For the assay and quantification of circulating RNAs as biomarkers, there is a need for robust normalizing controls to ensure the validity of the data. C.lin-4 has previously been reported and utilized as an exogenous control. Therefore, within this current investigation C.lin-4 was utilised to assess the quality of RNA extraction, reverse transcription and PCR. The excellent coefficient of variation (3.91%) confirmed that a consistent extraction, purification and amplification of the RNA had occurred. It has been suggested that an exogenously spiked-in RNA could be used for normalization. We have shown that synthetic miRNA degrades over time and as such it has limitations in its use. The use of endogenous controls allows correction for any differences in the quality of the starting material. U6snRNA and let-7d have been reported as endogenous control for miR-122 normalization. Within this study both the U6snRNA and let-7d also showed a low coefficient of variation. Within the published literature, the use of the

endogenous controls, such as U6snRNA and let-7d, as a normaliser is the subject of much debate. (180) Several different small RNA's and microRNA's have been suggested as the ideal normaliser. In reality, it has become apparent that there is no ideal normaliser and this subject is a rapidly evolving field and one that requires further investigation. Despite both U6snRNA and let-7d controls showing a low degree of variation, when applied as normalisers to the raw miR-122 data, they gave noticeably different results. The normalised miR-122 range by U6snRNA in particular was significantly larger than when normalised by let-7d. The difference in range could be attributed to the difference in Ct values between the normalizing controls and the target (miR-122). The let-7d has a mean Ct close to that of miR-122, leading to a narrow range, while the U6snRNA has a mean Ct value higher than miR-122 which resulted in a higher observed Δ Ct value. However, when normalized values were correlated at the level of the individual subject, they showed a strong and significant relationship and therefore either would be appropriate to use. In reality, it has been suggested that it would be appropriate to use a control with a Ct value closer to the miR-122, in this case let-7d. (180)

The use of % coefficient of variation to assess the variability of these molecules has both advantages and disadvantages. It has the advantage over standard deviation in that it is better when comparing data sets with widely different means, as seen in this experiment. However, its disadvantages lie in the fact that it can be sensitive to small changes in the mean and cannot be used to create confidence intervals. In this experiment, depending on the method used to express the data (Ct, normalised to control or copies/uL), there is a wide difference in the %CV. Measuring raw Ct may not be ideal as it is a log-scale and small variation can actually represent large differences as seen by the %CV of the normalised results. In reality, there remains a need for a standardised method of expressing data that is universally accepted.

A major limitation of PCR is that it is only semi-quantitative. Previously published reports have expressed their results either as raw Ct values (177) or normalised to a control. (173; 181) The difficulty is that the Ct value can vary depending on what threshold is selected for the baseline and subsequent analysis. Selecting the same threshold across an individual experiment allows comparison between the samples, but only within the experiment and cannot be compared to work carried out in a different laboratory, or even a different machine. The same applies for normalizing data, as the Ct values of the target and control are similarly variable. Differences in the method of RNA extraction, purification and PCR also mean that any data cannot be compared outside any particular experiment or between laboratories. Therefore, we sought to develop a method whereby robust comparison could be made independent of extraction technique or Ct threshold set at PCR. We have calculated, using a standard curve of synthetic oligonucleotide of known quantity, the number of copies of miR-122 per microlitre of serum in the healthy human. By expressing the data in this fashion, comparison of levels can be made across experiments and between research groups around the world, as the number of copies per microlitre method gives a definitive result independent of the extraction technique or PCR technique used. The widespread use of standardized and robust assays for the generation of circulating miR-122 data is important for the ongoing / rolling qualification of this analyte as a biomarker of liver injury and disease.

In conclusion, with this investigation we have shown that the hepatocyte specific microRNA, miR-122, is found in stable levels in the healthy human population. It has less variation than the current markers to assess liver integrity such as ALT activity. Furthermore, defining the normal reference range by copy number is the most accurate method of quantification and will allow inter-laboratory comparison between populations.

Chapter 3

Release of microRNA-122 in various pathological conditions in a human population.

3.1 Introduction

The potential use of microRNA-122 as a circulating serum biomarker for liver injury and toxicity is currently the focus of much investigation. Initial work mainly focused on drug toxicity in animal models with Wang et al showing in 2009 that mice receiving a toxic dose of paracetamol had significantly raised levels of miR-122 and that these levels rose before ALT. (169) The publication of a similar study in rats suggested that this effect might be seen across species. (170) Recently, work performed in the human population following acute paracetamol overdose (171) has shown that serum miR-122 levels are significantly raised as well as in a range of other conditions including hepatitis B and C infection (172; 173; 175) , hepatocellular carcinoma (174) and acute transplant rejection. (176)

The major limitation of these studies is the lack of multiple, different aetiologies of underlying disease to allow comparison. As discussed in the previous chapter, a cycle threshold value (Ct) cannot be used to compare between different experiments as the number is set specifically by the instrument used and makes no allowance for differing methodology. Thus, although all the conditions studied may lead to raised levels of miR-122, the relative differences between this variety of clinical entities cannot be assessed.

This chapter describes a comparison of levels of miR-122 release between groups of patients with several different liver diseases. This will allow a direct comparison of the miR-122 release in different types of injury. The levels of miR-122 will also be compared to traditional markers (AST) to assess the correlation between them. Previous work has shown that miR-122 and transaminases are well correlated following acute paracetamol hepatotoxicity. (171) Finally the potential role for miR-122 in the diagnosis of hepatocellular carcinoma will also be assessed as suggested in other studies. (173)

3.2 Methods

A total of 194 patients with (n=104) and without HCC (n=90) and with a variety of chronic liver diseases enrolled along with healthy controls as part of a study to develop a novel panel of biomarkers for primary liver cancer. (Ethics REC reference 06/Q2707/182). The patients were stratified into 10 groups according to disease type (Table 11). The median age of the entire cohort was 61 (range 35-88). Patients were recruited from the Hepatology clinic and cirrhosis was diagnosed by a standard combination of clinical presentation, imaging and biopsy.

	HCC +ve	HCC -ve
No liver disease	24	19
Hepatitis B Virus	21	18
Hepatitis C Virus	21	18
Alcoholic Liver Disease	19	19
Other causes of cirrhosis	19	16
Total	104	90

Table 11: Patients stratified according to underlying liver disease

MicroRNA-122 levels were analysed by RT-PCR using the methods described in the previous chapter. To avoid any concern about degradation during sample collection or storage, an endogenous control small nuclear RNA (U6snRNA) was measured as a normaliser with the results expressed as $\Delta\text{Ct miR-122/U6snRNA}$.

AST results were provided from the trial database in Birmingham. Statistical analysis was carried out using GraphPad Prism with ANOVA and contingency table to define differences between groups, correlation to compare miR-122 and AST and receiver operator characteristic (ROC) curves calculated to assess sensitivity and specificity of the test. Statistical significance was set at $p=0.05$.

3.3 Results

3.3.1 Analysis of miR-122 levels between groups

The levels of miR-122 were assessed between the different groups. The group of patients with liver disease had a significantly raised serum miR-122 ($p < 0.001$) than the normal population as shown in Figure 36

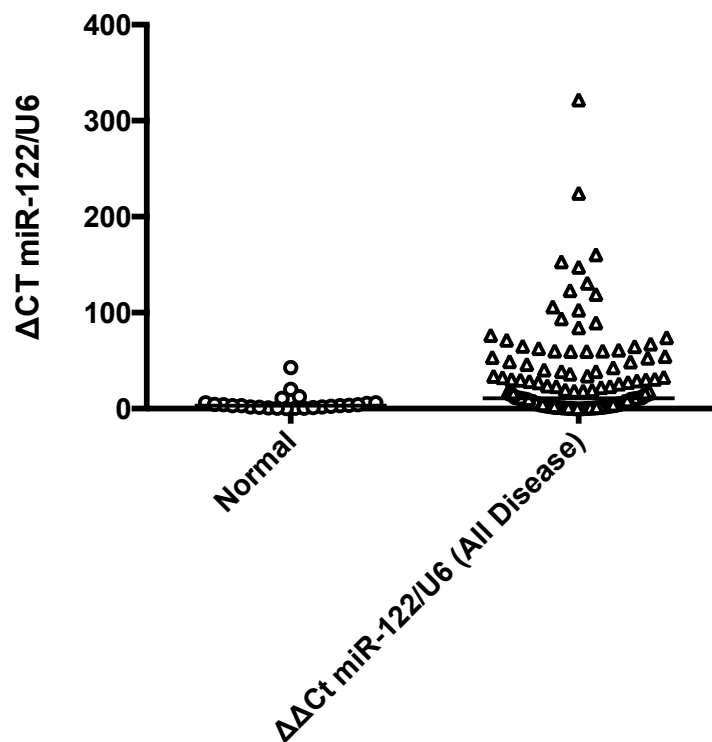


Figure 36: Comparison of serum miR-122 between normal and disease groups. There was a significant difference ($p < 0.001$) between the group with liver disease and the healthy controls.

When the groups were analysed for differences between groups, four were seen to be significantly higher than normal (Figure 37). These were the alcohol, HBV, HCV and HBV with HCC groups. (Table 12)

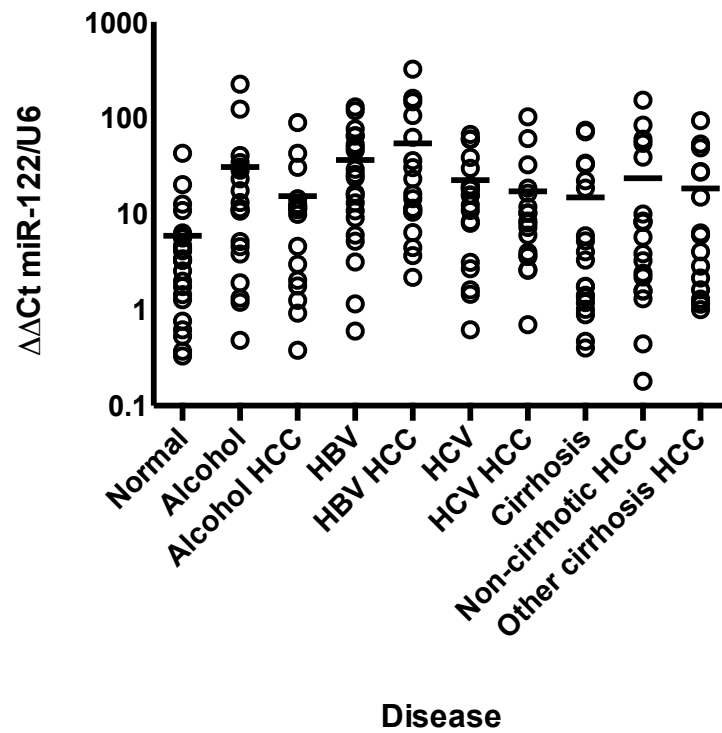


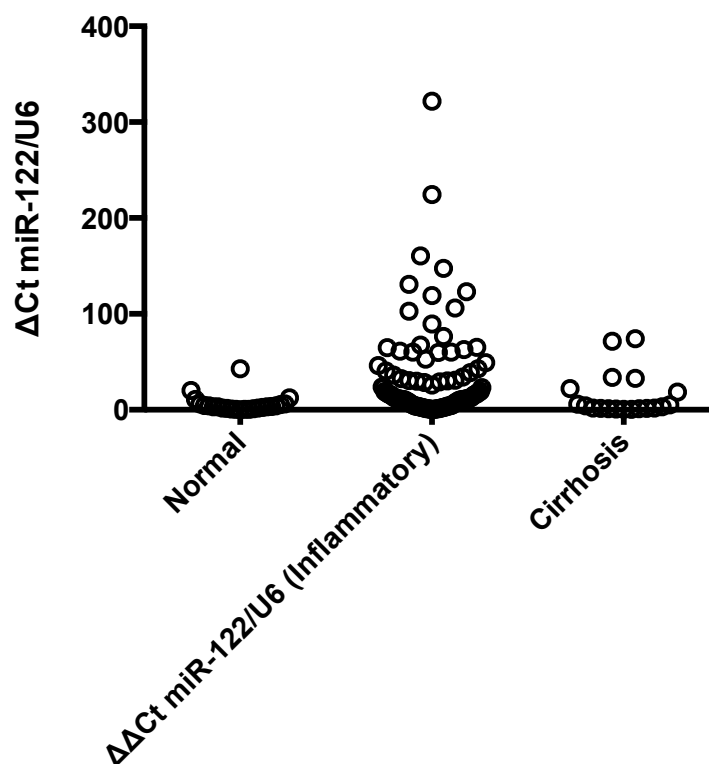
Figure 37: Serum miR-122 levels in the different groups. There is significant overlap between the groups probably reflecting the heterogeneity of the patients disease state.

Liver Disease/Condition	P value
HBV related HCC	0.0002 *
HBV	0.0004 *
HCV	0.0112 *
Alcoholic Liver Disease	0.0349 *
HCV related HCC	0.1328
Alcoholic Liver Disease related HCC	0.4215
Other cirrhosis related HCC	0.4894
Non-cirrhotic HCC	0.4911
Cirrhosis	0.9630
Normal	1.0

Table 12: Summary of individual groups. Only 4 groups had a significantly raised level of miR-122 (marked by *). These were the conditions causing hepatitis and inflammation rather than those with no inflammation.

Patients with conditions with an inflammatory aetiology were compared to those with an underlying cirrhotic condition. Those with inflammatory

conditions were shown to have significantly higher serum miR-122 levels compared to both normal and cirrhosis, while those with cirrhosis has levels similar to normal. (Figure 38)



Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Inflammation	Normal	1.525009	0.3484751	0.833729	2.216290	<.0001 *
Inflammation	Cirrhosis	1.126770	0.3799584	0.373035	1.880505	0.0038 *
Cirrhosis	Normal	0.398240	0.4441039	-0.482743	1.279222	0.3720

Figure 38: Comparison between normal, inflammatory and cirrhotic conditions. There were significant differences in serum miR-122 between the group with inflammation and the controls ($p < 0.001$) and those with no inflammation/cirrhosis ($p = 0.0038$). There was no difference between the controls and those with cirrhosis without inflammation ($p = 0.372$).

3.3.2 Serum miR-122 and serum AST levels.

Comparison of serum miR-122 showed no correlation to serum AST (Pearson $r = 0.06$, $p = 0.45$) as shown in Figure 39. Sub-group analysis of individual groups showed no correlation in any group, although the group

with cirrhosis had a weak but not statistically significant correlation ($r=0.56$) (Table 13).

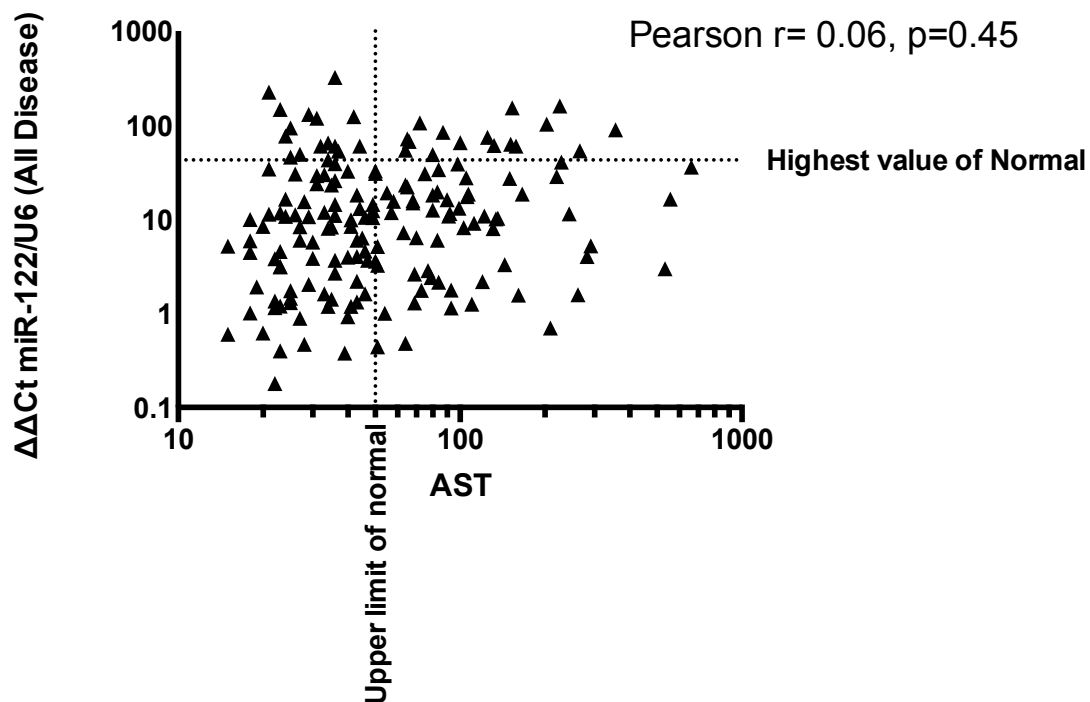


Figure 39: Correlation of serum miR-122 with serum AST

Correlation between log($\Delta\Delta\text{Ct}$) and log(AST)	
Overall	0.20
Alcohol	0.20
Alcohol HCC	0.13
Cirrhosis	0.56
HBV	0.37
HBV HCC	0.12
HCV	0.39
HCV HCC	0.23
Non-cirrhotic HCC	0.27
Normal	
Other cirrhosis HCC	0.21

Table 13: Correlation between serum miR-122 and serum AST by group showing very little in the way of correlation between serum miR-122 and AST.

Receiver operator characteristic (ROC) curves were generated to compare serum miR-122 and serum AST for use in the diagnosis of inflammatory or cirrhosis damage to the liver.

In the first instance the sensitivity of miR-122 to detect the 2 types of damage was calculated. MiR-122 was better at detecting inflammatory damage with an Area Under the Curve (AUC) of 0.79 ($p<0.001$) than cirrhosis (AUC 0.54, $p=0.36$) as shown in Figure 40.

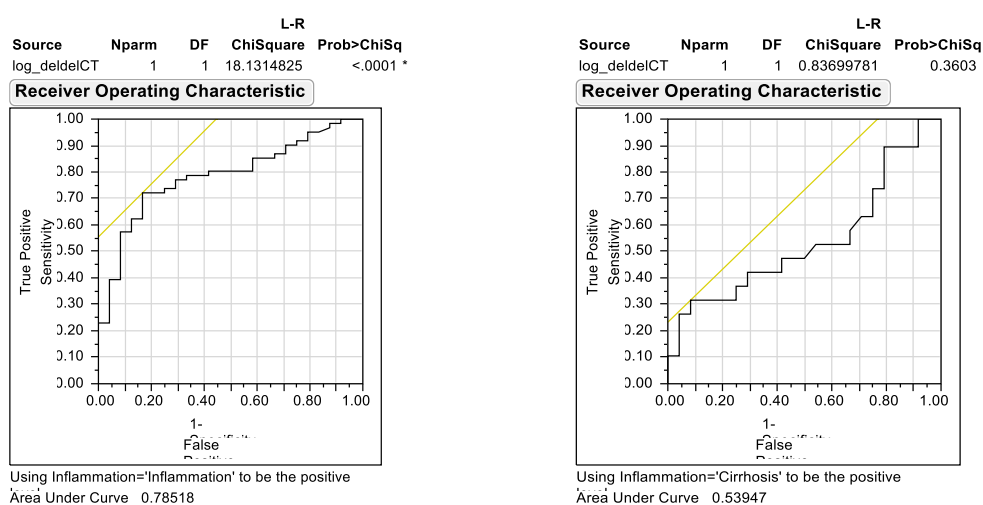


Figure 40: ROC curves of miR-122 to detect inflammatory change versus cirrhotic change. MiR-122 seems better at detecting patients with inflammatory conditions (AUC 0.79) than those with non-inflammatory cirrhosis (AUC 0.54). However, the sensitivity at 90% specificity is still low in the inflammatory group just under 60%.

ROC curves were generated to assess the value of serum miR-122 and serum AST at differentiating between different types of liver injury. Serum miR-122 was better than AST at detecting the difference between inflammatory disease and cirrhosis (AUC 0.68 vs 0.56), but combining the 2 markers into a panel was better than either individually (AUC 0.74) as shown in Figure 41.

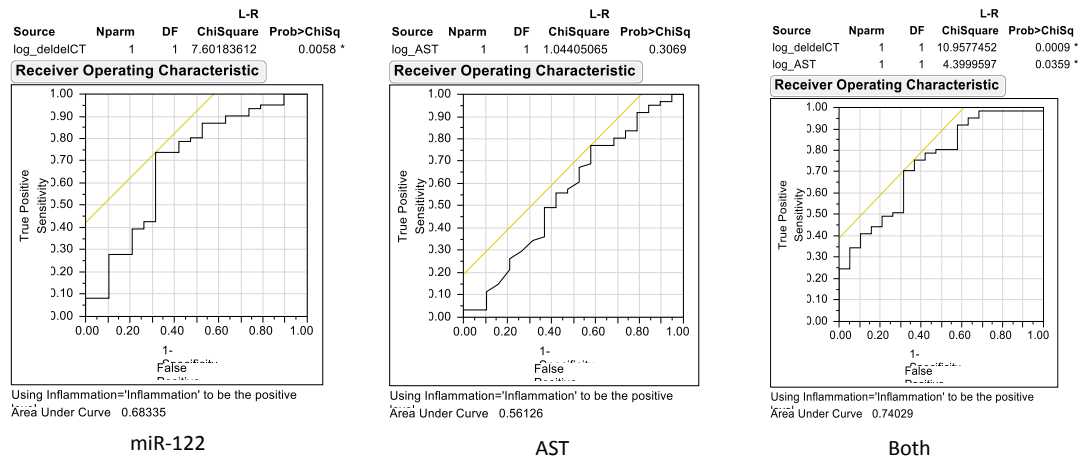


Figure 41: ROC curves of serum miR-122 and AST in detecting between inflammatory damage and cirrhosis. By combining the two biomarkers, the test become better (AUC both 0.74 vs 0.68 (miR-122) and 0.56 (AST)). Despite this the sensitivity at 90% specificity is low at around 35%.

3.4 Discussion

There is potential for serum miR-122 to be used as a biomarker for liver disease and injury. While previous studies have compared only a single condition with normal controls, in this study nine distinct clinical conditions were compared against a normal population. Patients with liver disease had significantly higher serum miR-122 levels than that of a normal population. This supports previous work reported in the literature (172-176). It has also shown that within the liver disease population that the serum miR-122 levels are affected by the aetiology of the disease, in particular, those patients with an inflammatory process (alcohol liver disease, HBV & HCV) have a much higher level, whereas with established cirrhosis of the liver, levels are comparable to the normal population. This result is not unexpected, as miR-122 is an hepatocyte-specific microRNA and is released when there is hepatocyte damage as seen in conditions causing a hepatitis of the liver. The low levels of serum miR-122 in patients with cirrhosis have been previously shown in patients with decompensated alcoholic liver cirrhosis (177). This may reflect the fact that cirrhotic change is associated primarily with fibrosis and scarring of the liver, rather than direct

hepatocyte damage, resulting in little release of hepatocyte-specific markers into the bloodstream. A second possibility is that by the time established cirrhosis is present, liver scarring means that there are fewer normal functioning hepatocytes remaining to release miR-122 in to the circulation resulting in the low-normal levels. It may be that serum miR-122 will be a good biomarker for certain conditions but not as good for others.

Serum transaminases have been shown to correlate well with histopathological liver damage (168) and previous work in our group in patients with acute paracetamol overdose has shown good correlation between serum miR-122 and serum ALT (171). In this study there was no correlation between serum miR-122 and AST. A possible explanation may be that most other studies involve late-stage damage to the liver. In the previous studies published in animal models, most are given a toxic dose of drug (169). In the human paper published by our own group (171) the patients with acute paracetamol overdoses had significant hepatic necrosis requiring admission to a liver transplant unit. It is known that serum miR-122 rises are detectable before the serum transaminases rise. Elfimova et al suggested that before miR-122 and transaminases spill out of a disrupted cell membrane into the bloodstream that there is release of miR-122 from intact cells via another, as yet undefined mechanism. (182) This position is strengthened by work from Bala et al, who have shown that miR-122 is actively released from cells under stress. (183) Given that our population represents a cohort of patients living at home and attending outpatient clinic, they do not fall into the end-stage category. It is possible that the lack of correlation between serum miR-122 and AST is as a result of active release of miR-122 from a viable cell prior to the collapse of the cell membrane leading to spilled transaminases. If so this could hint at a potential, important role for miR-122 in the early diagnosis of liver disease and injury.

A major limitation of this study is the lack of more clinico-pathological detail on each of the patients. Such information would provided more insight into

the extent of disease rather than just a “diagnosis” and in an ideal world this data would have been available.

Currently the gold standard for liver disease is a panel of biomarkers including transaminases, serum bilirubin and other liver enzymes (alkaline phosphatase and gamma-glutamyl transpeptidase). MicroRNA-122 has been suggested as an alternative for this current panel. The ROC analysis carried out confirms that miR-122 is better at differentiating between types of liver damage than AST. However, when both were combined together, the sensitivity of the test increased. It is likely that in the future, the use of serum miR-122 will become more mainstream in the diagnosis of liver disease. However, it is likely that it would best be used as part of a panel of biomarkers in conjunction with current tests rather than a single diagnostic for detecting liver injury.

The use of ROC curves to express the value of a biomarker has both advantages and disadvantages. They allow a representation of the sensitivity and specificity of each individual sample rather than a summary of the whole cohort. The use of AUC tells us how good the test really is. Caution should be used, however, as AUC can be misleading in certain circumstances. Two tests may have the same AUC, but one may actually be better than the other. The discriminator is the “sensitivity at 90 or 95% specificity”. The higher this is, the better a test is.

Serum miR-122 levels are significantly raised when there is hepatitis, but not so when the primary pathology is cirrhosis. Irinotecan and oxaliplatin are two of the main drugs used in the treatment of metastatic colorectal cancer. As mentioned previously, irinotecan is associated with the development of steatohepatitis while oxaliplatin is associated with fibrosis and the development of sinusoidal obstruction. Given the results here, it may be that serum miR-122 will have more of a role in detecting irinotecan damage over oxaliplatin damage to the liver. This hypothesis will be explored in the succeeding chapter.

Chapter 4

MicroRNA-122 as a potential biomarker for
hepatotoxicity from neo-adjuvant chemotherapy
for colorectal cancer liver metastases.

4.1 Introduction

Patients with liver metastases from primary colorectal cancer are often considered for neo-adjuvant chemotherapy as part of their treatment. In those with irresectable or borderline resectable disease, tumour response may allow resection and improve survival. (59; 61) It also identifies those in whom the disease is progressive and in whom surgery would be futile. (51) The publication of the MAGIC trial in 2006 showed the potential benefit of neo-adjuvant chemotherapy in resectable gastrointestinal cancer. (50) It was felt that this approach might be beneficial in colorectal liver metastases, particularly as it may treat micro-metastases within the liver. A large-scale trial was set up to investigate this. (56)

Chemotherapy-induced hepatotoxicity affected up to 78% of patients receiving standard chemotherapy for colorectal cancer and can lead to increased morbidity and mortality. (179) Hepatotoxicity presents a challenge both for the treating oncologist and the operating surgeon in how to manage the impact on a successful outcome.

Increasingly, more aggressive chemotherapy regimens are being trialed in an attempt to improve survival outcomes. These include increasing the number of agents used in combination and increasing the duration of chemotherapy. Triple therapy with 5-FU, oxaliplatin and irinotecan (FOLFOXIRI) has been shown to significantly increase PFS but at the cost of increased toxicity. (184) Similarly, extended treatment (>9 cycles) has shown no survival benefit but also leads to increased toxicity. (185)

There remains a need to diagnose significant hepatotoxicity pre-operatively. It remains unclear what functional impact chemotherapy-induced hepatotoxicity has however expert opinion is that more future remnant liver should be left to allow for the potential of impaired function. (104) Thus any change in the planned operative strategy would require pre-operative identification of hepatotoxicity. Currently, patients receiving chemotherapy

have blood sampling at each cycle measuring the liver enzyme ALT as a marker for hepatic damage. Unfortunately, patients continue to present at laparotomy with chemotherapy-damaged liver despite having normal blood tests during treatment. A new biomarker is urgently needed to allow personalised therapy for individual patients.

The use of microRNA-122 as a more specific and sensitive marker of liver injury is currently the subject of much research. Following on from the previously discussed literature, a pilot study was designed to assess the potential role of this novel biomarker in the pre-operative diagnosis of chemotherapy-induced hepatotoxicity.

4.2 Methods

4.2.1 Patient Recruitment

Patients were recruited under the National Ethics Research Service approved study “Biomarkers for hepatotoxicity of neoadjuvant chemotherapy for colorectal liver metastases: a proof of principle study designed to evaluate the clinical utility and value of novel serum markers” (REC ref. 11/NW/0709). Patients due to receive neo-adjuvant chemotherapy prior to surgical resection of colorectal cancer liver metastases were identified through the Merseyside and Cheshire Supra-regional Specialist Multi-disciplinary meeting (sMDT) at University Hospital Aintree, Liverpool. Patients who had received previous chemotherapy were excluded. Suitable patients were approached and given a REC-approved Patient Information Sheet (PIS). Those who were willing to take part were consented and entered into the study as shown in the Study Protocol in Figure 42.

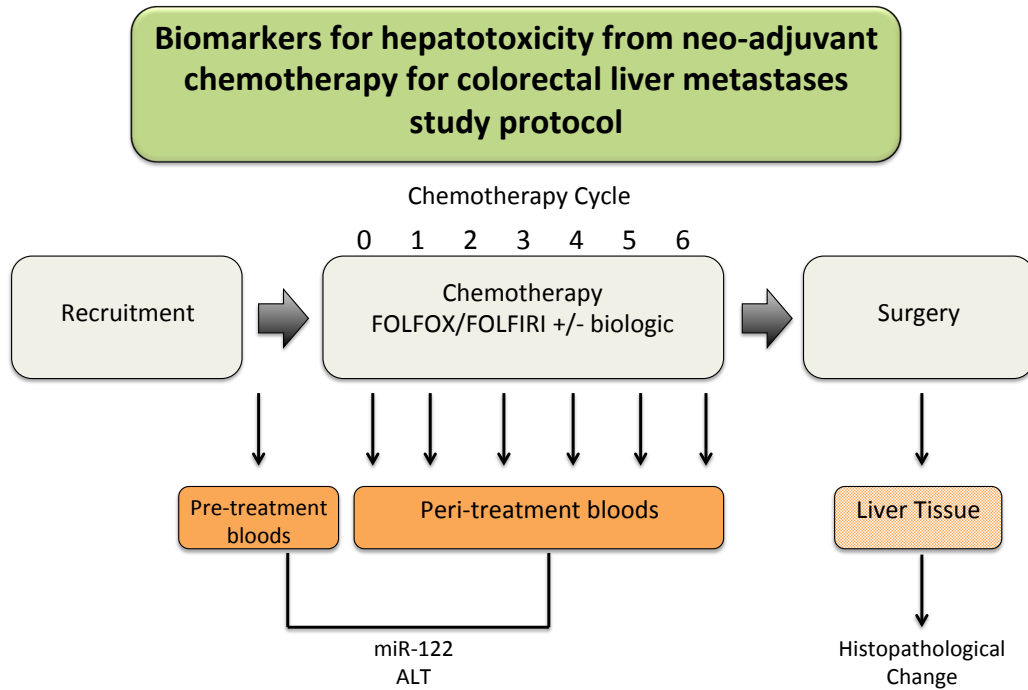


Figure 42: Study Protocol for Pilot Study. Patients had blood samples taken routinely prior to each cycle of chemotherapy and liver tissue sampled at the time of surgery.

Patients were recruited over an 18-month period at 3 centres: University Hospital Aintree, Royal Liverpool and Broadgreen University Hospital and Clatterbridge Cancer Centre. Having been identified in the sMDT at University Hospital Aintree. Patients were then approached in the Liver Clinic at UHA and the project was explained to them. If they agreed to take part, consent was taken prior to the start of chemotherapy.

Serial blood samples were obtained as part of their standard treatment both before the commencement of chemotherapy and before each treatment cycle. At this time an extra 5ml of blood was taken for measurement of serum miR-122.

4.2.2 Sample analysis

ALT was measured in the respective NHS hospital laboratory in the standardised fashion for that particular hospital. The extra sample was

centrifuged at 1300g for 10 mins at 4 degree Celsius and the serum stored at -80 degrees Celsius until analysis.

MiR-122 levels were measured by qRT-PCR as described in Chapter 2. The results were expressed as both Ct value and number of copies/uL of serum.

Histopathological analysis of the background liver parenchymal tissue was conducted by a Consultant Histopathologist at University Hospital Aintree from the resected specimen. Both the Consultant and myself graded it according to the Brunt and Rubbia-Brandt scoring systems.

4.2.3 Statistics

Statistical analysis was carried out using GraphPad Prism (GraphPad Software, La Jolla, CA) for analysis of variance (ANOVA) and using SAS (SAS Institute Inc, Cary, NC, USA) for least squared means analysis. Statistical significance was set at $p=0.05$.

4.3 Results

Eleven patients were recruited in the 18-month period between February 2012 and July 2013. Of these, 10 successfully completed their neo-adjuvant chemotherapy and proceeded to liver resection for their metastases. One patient presenting with locally advanced synchronous disease failed to tolerate the chemotherapy and after 4 cycles it was abandoned due to ongoing sepsis from a scrotal abscess and poor tolerance of the prior cycles. Unfortunately, disease progression in both sites made the aim of treatment purely palliative.

As expected the majority of patients were those with advanced (T3/4) primary tumours and multiple liver metastases. The majority received first line therapy with an oxaliplatin and 5-FU/capcitabine based regime in keeping with NICE guidelines. Unfortunately by the end of the study period,

60% of the resected patients had developed recurrence at a median interval of 6 months post-operatively and 1 had sadly passed away from their disease. The full demographics of the patients are shown below in Table 14

Histopathological analysis of the resected livers showed that all ten resected patients had some form of potential chemotherapy-related injury to their liver. (Table 15)

Patients	Recruited	N= 11
	Underwent Liver Resection	10 (91%)
	Age (median)	64 (47-80)
	Male	8 (73%)
	Female	3 (27%)
Presentation	Synchronous	9 (82%)
	Metachronous	2 (18%)
	Time elapsed to metachronous presentation	16-33 months
Primary Colorectal Tumour Stage	T1	0 (0%)
	T2	1 (10%)
	T3	6 (60%)
	T4	3 (30%)
	N0	3 (30%)
	N1	4 (40%)
	N2	3 (30%)
	M0	2 (20%)
	M1	8 (80%)
Number of Metastases	Solitary	3 (27%)
	Multiple	8 (73%)
Chemotherapy Regime	FOLFOX/OXMdG	5 (45%)
	CAPOX	5 (45%)
	FOLFIRI/IRIMdG	1 (10%)
	+ Cetuximab	3
Recurrence	Yes	6 (60%)
	No	4 (40%)
	Time to recurrence (median)	6 months (1-13)
Mortality		1 (9%) at 20 months post-op

Table 14: Patient demographics from “Biomarkers for Hepatotoxicity” pilot study

Patient	Steatosis	Steatohepatitis	Sinusoidal Obstruction
1	Mild	Mild	Mild
2	Severe	Mild	Mild
3	Mild	Normal	Mild
4	Mild	Normal	Normal
5	Severe	Mild	Mild
6	Moderate	Normal	Normal
7	Severe	Mild	Normal
8	Normal	Normal	Mild
9	Mild	Normal	Moderate
10	Mild	Mild	Mild
11	Not resected		

Table 15. Histology of resected post-chemotherapy liver by patient. All 10 resected patients had evidence of histological change that could potentially be chemotherapy related.

The baseline miR-122 was within the range of that seen in the normal patients in all nine patients for whom a baseline blood sample was available. (Figure 43)

Over the course of the chemotherapy treatment a slight increase in the mean miR-122 levels was seen (Figure 44), however, analysis of variance (ANOVA) showed that this was not significant at any individual cycle.

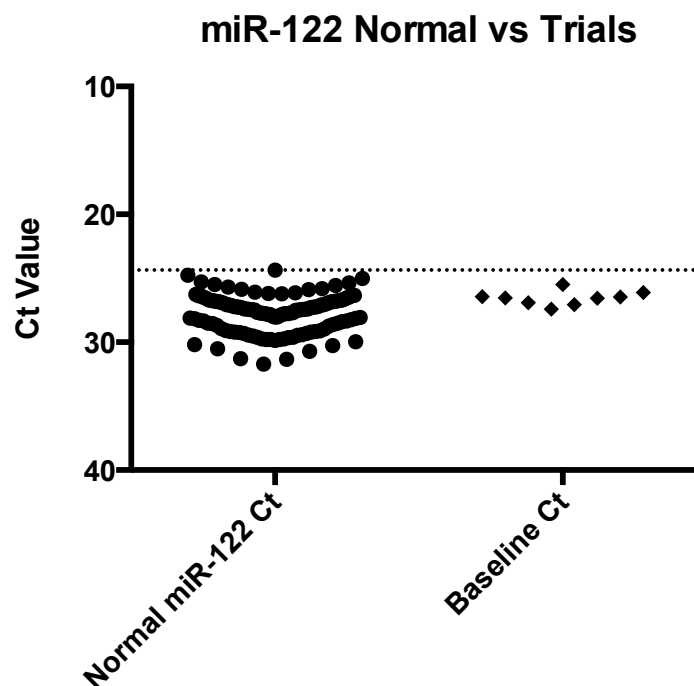


Figure 43. Baseline miR-122 levels in the study patients within the same range as the normal patient population that was defined in Chapter 2.

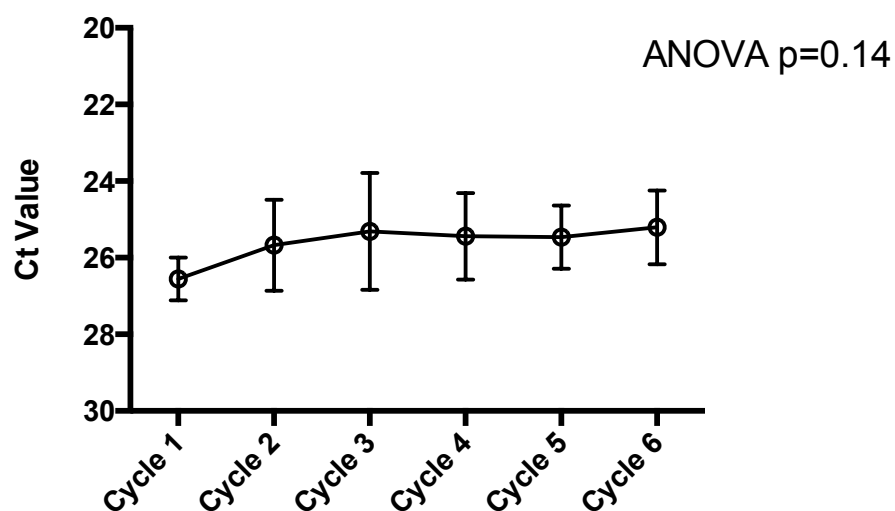


Figure 44. miR-122 levels for all patients throughout chemotherapy treatment. Although there was a trend to a rise in serum miR-122, there was no significant difference ($p=0.14$).

The paired ALT and miR-122 samples for all patients were analysed and showed a strong correlation ($r=0.75$, $p<0.001$) as shown in Figure 45.

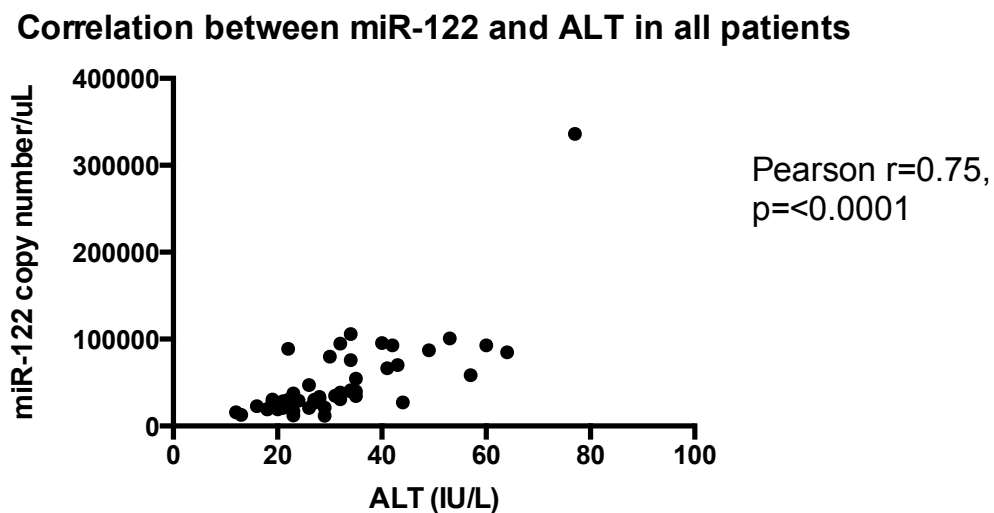


Figure 45. Correlation between miR-122 and ALT in all patients showing good correlation ($r=0.75$, $p<0.001$).

The potential utility of serum miR-122 and ALT to detect hepatotoxicity was compared to the histopathological liver change at the time of surgery using statistical models of repeated measures analysis and least squared means of the results. The sensitivity of the statistical analysis was limited by the low power of this pilot study. The patients were divided into two groups for analysis depending on the histology of the resected sample. The steatosis group was divided into normal/mild/moderate vs severe, the steatohepatitis group into normal vs mild and the sinusoidal obstruction group into normal vs mild/moderate.

The results are shown in Figures 46-48. In each of the three types of hepatotoxicity, there was a statistically significant, but clinically insignificant rise in ALT during chemotherapy treatment. Although serum levels of miR-122 tended to rise this was not statistically significant. Neither ALT nor serum miR-122 were able to predict the end pathological damage of the chemotherapy.

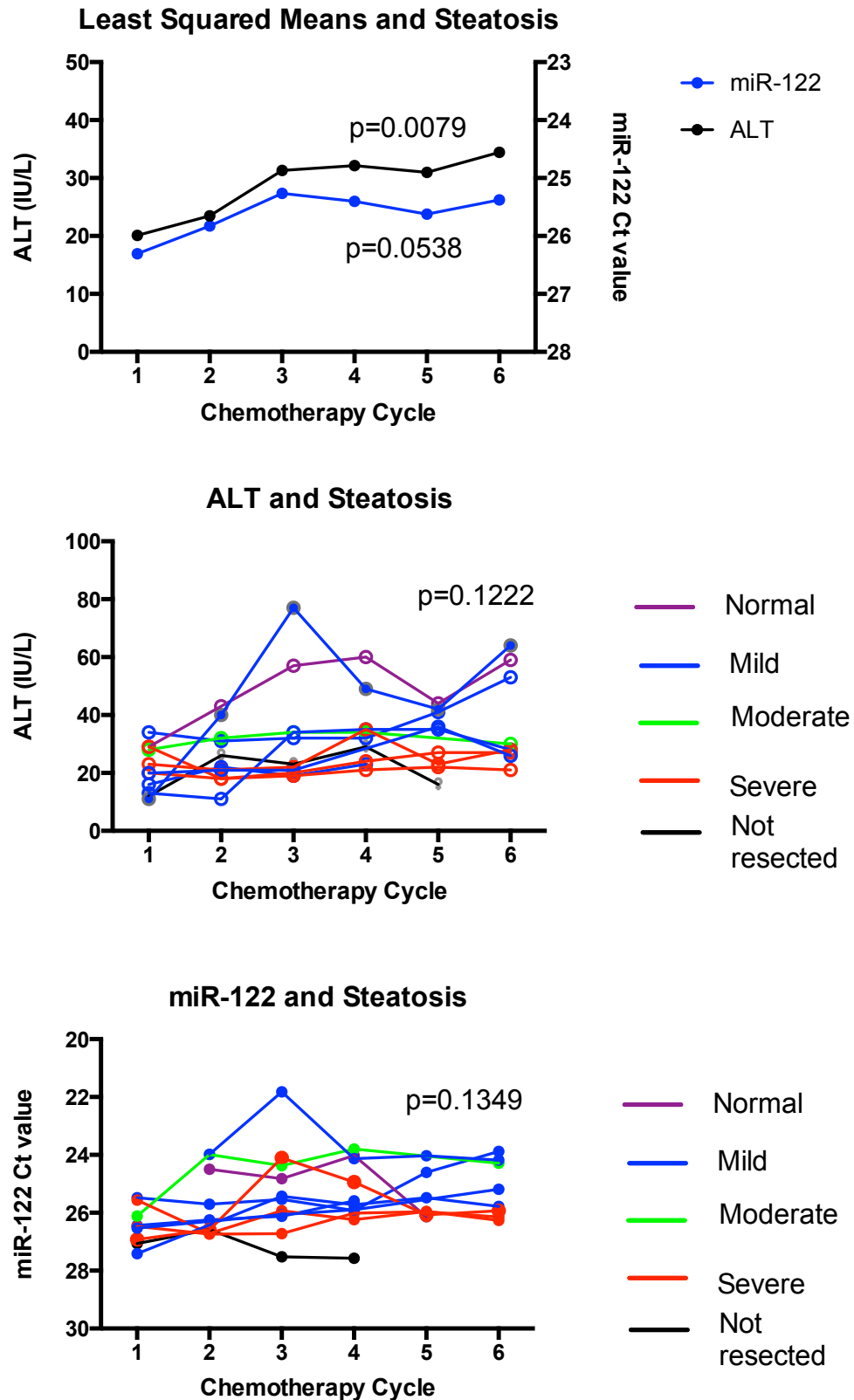


Figure 46. Comparison between ALT and miR-122 in patients with steatosis. There was a statistically significant but clinically insignificant rise in ALT ($p=0.0078$) but no rise in miR-122 ($p=0.0538$).

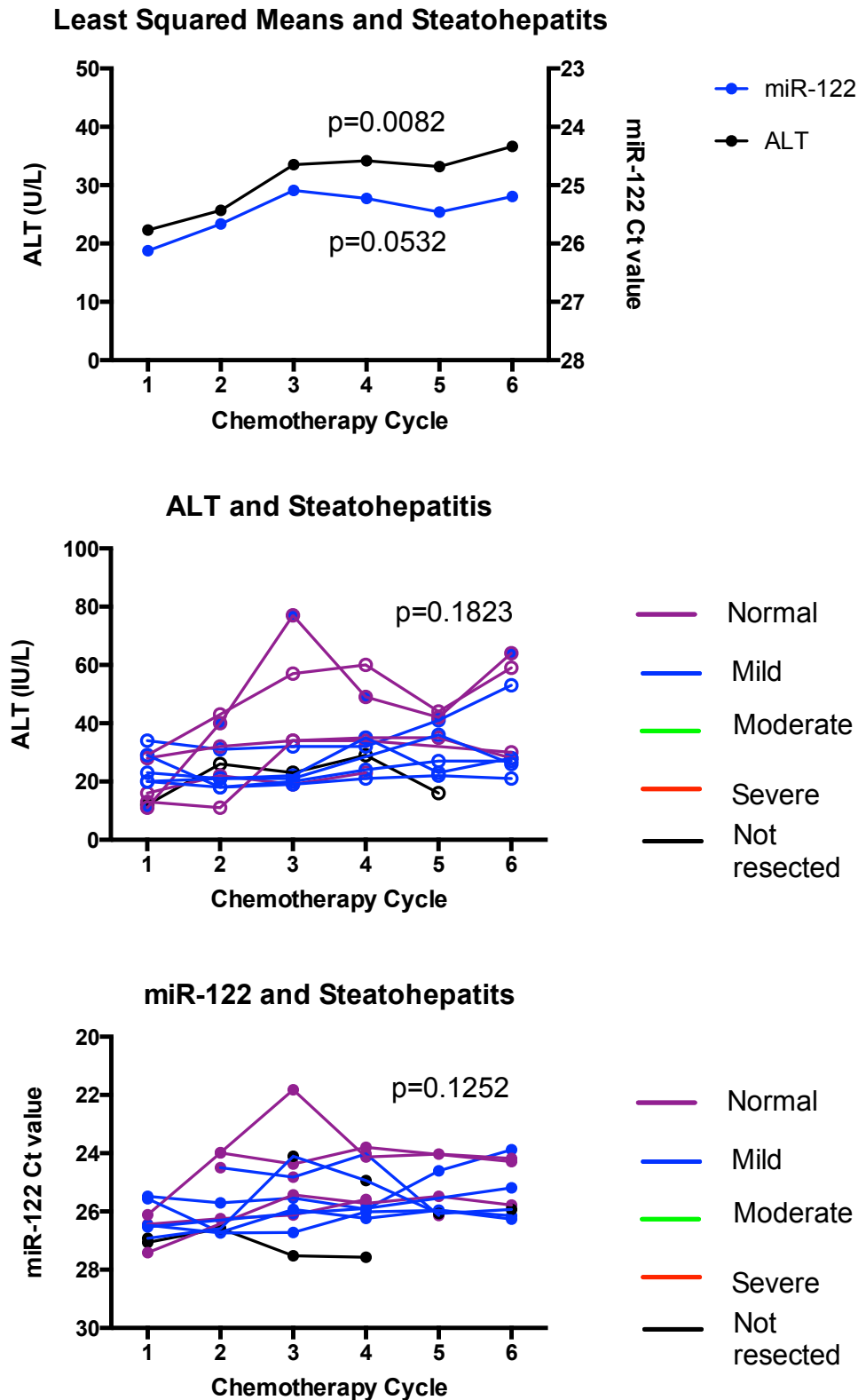
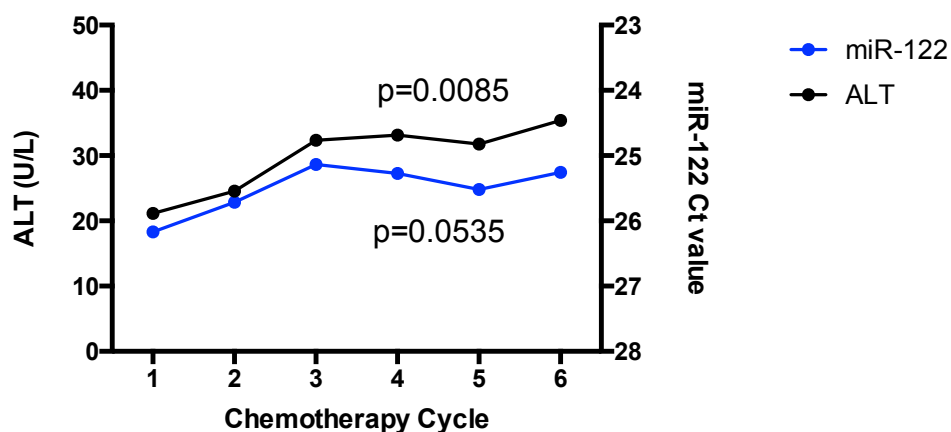
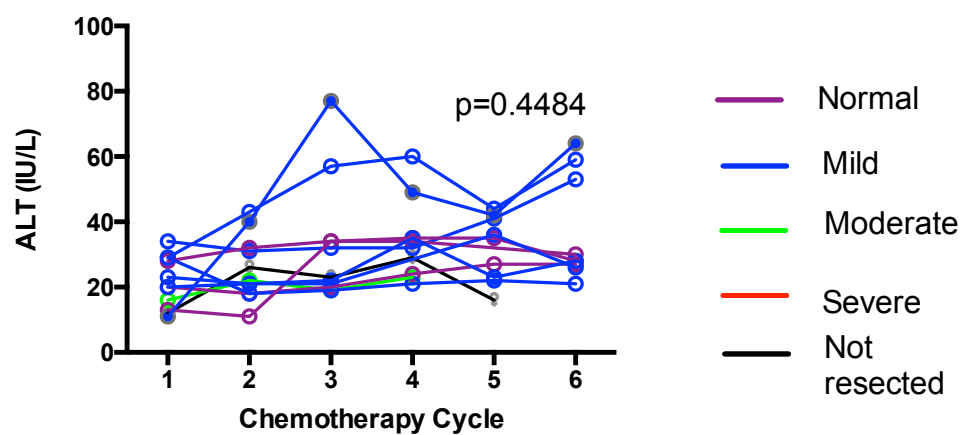


Figure 47. Comparison between ALT and miR-122 in patients with steatohepatitis. There was a statistically significant but clinically insignificant rise in ALT ($p=0.0082$) but no rise in miR-122 ($p=0.0532$).

Least Squared Means and Sinusoidal Obstruction



ALT and Sinusoidal Obstruction



miR-122 and Sinusoidal Obstruction

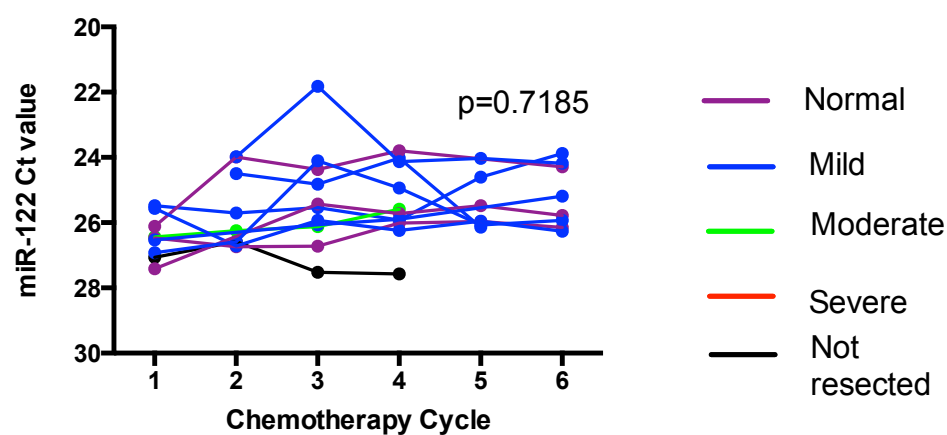
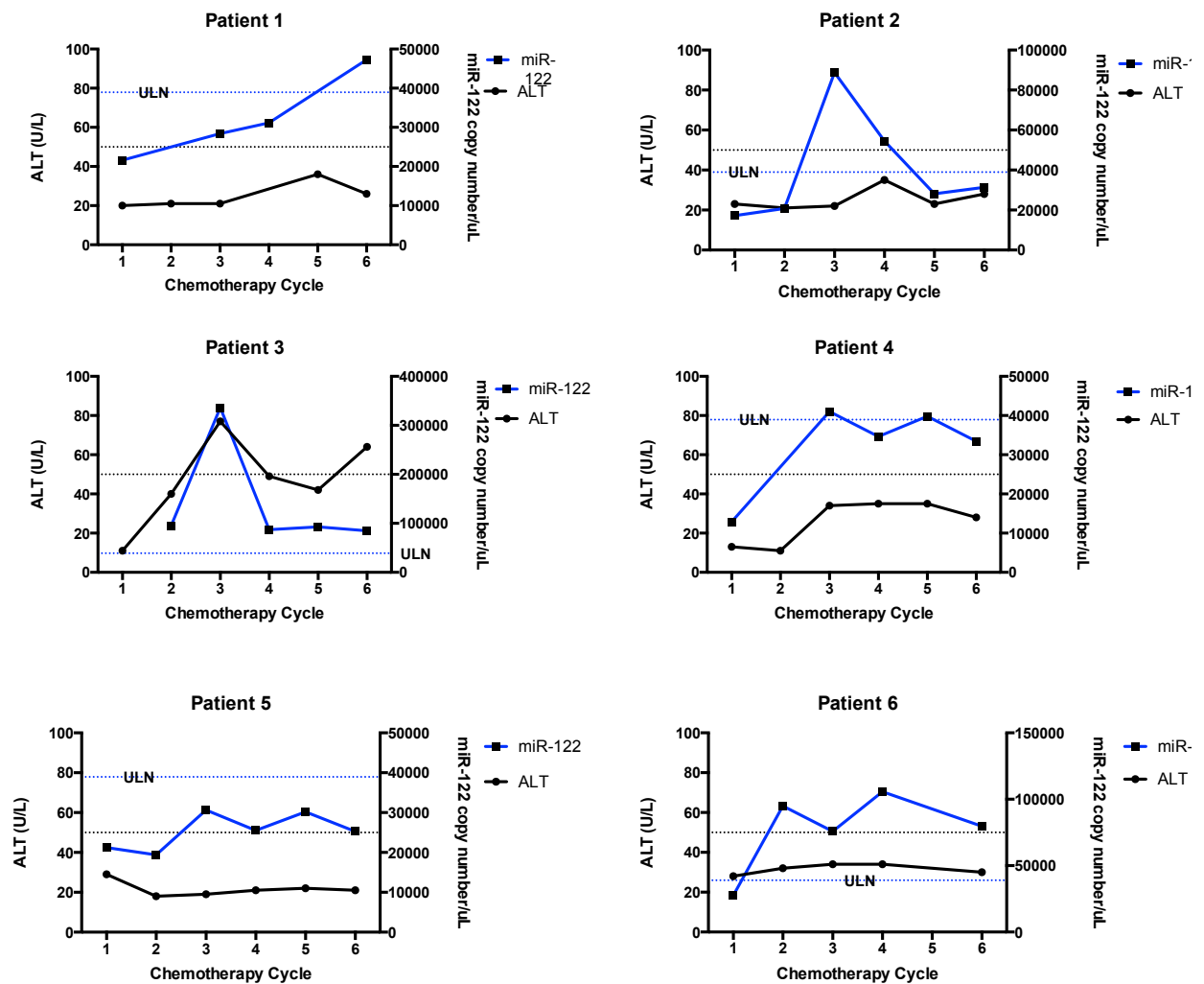


Figure 48. Comparison between ALT and miR-122 in sinusoidal obstruction syndrome. There was a statistically significant but clinically insignificant rise in ALT ($p=0.0085$) but no rise in miR-122 ($p=0.0535$).

When the ALT and serum miR-122 results were compared for each patient it appeared that they followed a similar pattern, although it would seem that the miR-122 levels generally rise before the ALT does. Due to the low numbers it was not possible to perform statistical analysis on these and so they remain descriptive observations. (Figure 49).



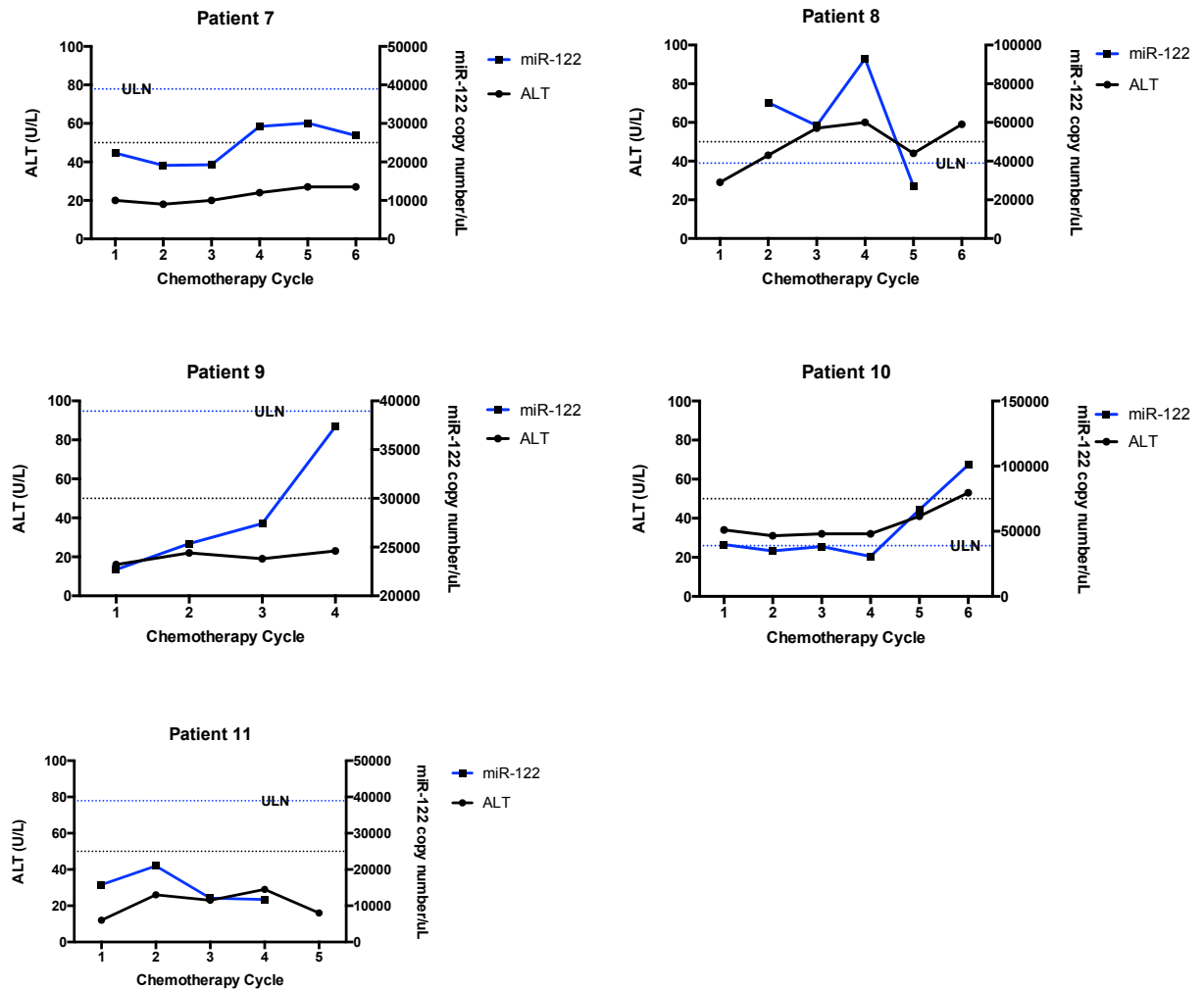


Figure 49. ALT and miR-122 levels for each individual patient for each cycle of chemotherapy. There is a suggestion of a relationship between the miR-122 and the ALT as the curves appear similar in most patients although the miR-122 changes tend to be seen a cycle or two earlier than the ALT.

4.4 Discussion

Chemotherapy-induced hepatotoxicity remains a significant event in a subgroup of patients receiving chemotherapeutic agents for advanced colorectal cancer. The ideal biomarker would be easy and safe to measure and be able to detect damage at a point before any clinically significant damage was done allowing therapy to be altered or terminated on an individual patient basis.

Based on the early literature, miR-122 appears to be a good candidate to fulfil this role. This pilot study was carried out to assess the potential role for miR-122 in this particular clinical situation.

The pilot study showed no significant association between the miR-122 and the final histology of the resected liver. All patients had some form of histological finding (steatosis, steatohepatitis or sinusoidal obstruction) that is found in association with chemotherapy treatment for advanced colorectal cancer. A non-significant trend to increasing levels of miR-122 over the course of the chemotherapy was seen but it does not predict the degree of the end pathological change. A statistically significant, but clinically insignificant, rise in ALT over the course of chemotherapy was seen. Moreover, in 7 of the 11 patients in the pilot, the level of miR-122 was at some point in the treatment over the range previously calculated as the upper limit of normal in a normal, healthy population.

Based on these findings, the study has proved the Null Hypothesis and has shown that there appears to be no role for miR-122 in the diagnosis of chemotherapy-induced hepatotoxicity in the treatment of advanced colorectal cancer.

However, the low recruitment to the pilot of only 11 patients of which only 10 had liver histology available raises the potential for a Type II statistical error to have occurred. It is possible that due to the low numbers a false negative result has occurred and the Null Hypothesis has been incorrectly accepted. Despite the low numbers, there are a few interesting findings from the pilot.

Despite the presence of liver metastases, other medical conditions and prescription drugs that could affect the liver, the baseline miR-122 for all patients who had a baseline sample available was within the limits of the “normal population” we had defined in an earlier experiment. (Figure 44) This could suggest that miR-122 is associated more with acute rather than

chronic disease or injury processes and as such may still have a role to play in acute chemo- or drug-induced toxicity.

MiR-122 was shown to correlate well with ALT, a finding that has been shown previously in humans by Starkey Lewis et al from our own research group. (171) The graphs of the individual patients results seem to visually confirm this as the profiles of the graphs are very similar. What is noticeable in several of the cases is that the miR-122 seems to rise a cycle or so before the ALT, again mirroring previously published results by Starkey Lewis that miR-122 is more sensitive than ALT. Elfimova et al have suggested that miR-122 is released before the cell membrane is damaged by other as yet undescribed processes. (182)

It is also possible that the end histology does not solely report chemotherapy-induced hepatotoxicity in these patients. Steatosis is very common in the general population. It is found in 10-24% of normal individuals and in up to 74% in obese patients. (186) The lack of a pre-chemotherapy liver biopsy means that we cannot say for certain that the steatosis found was not present at the start and therefore the fact that there was no rise in the serum miR-122 reflects that there was no new damage to detect. Steatohepatitis and sinusoidal obstruction are less common and more directly associated with chemotherapy. Most patients showed only mild or no sign of these pathologies, again suggesting a potential explanation for the non-significant changes in miR-122. Although not seen in this pilot study, a potential limitation of the design may be that patients who develop significant hepatotoxicity may stop therapy early and not proceed to surgical resection. This could lead to their exclusion from the pilot and the potential of important data being missed.

4.5 Conclusion

The results of the pilot study do not confirm a role at present for the use of miR-122 as a biomarker for chemotherapy-induced hepatotoxicity in the

treatment of advanced colorectal cancer. However, the low numbers involved and the technical limitations of the pilot study may have led to a false negative outcome being reported. In keeping with the increasing weight of evidence for miR-122 in the literature and some of the results shown here, we believe that more work is needed and that a potential role for miR-122 in this field may still exist.

Chapter 5

General Discussion

5.1 Introduction

Hepatotoxicity associated with chemotherapy is likely to increase as the choice and availability of novel drugs becomes greater. With increasingly powerful new agents and different combinations, toxicity and side-effects become more of an issue. There remains a need for better biomarkers in general for the detection of drug-induced liver injury, but particularly in chemotherapy, where significant side-effects may affect the intended benefit of the drug in terms of quality of life and outcome.

5.2 MicroRNA-122 as a biomarker for hepatotoxicity

The need for more sensitive and specific biomarkers of hepatotoxicity associated with cancer chemotherapy has increased over the last ten years due to increased choice and increased use. In particular, miR-122 holds considerable promise in a number of clinical areas. Recently, there have been a number of key papers published involving human patients rather than animal models. Within our own group, Starkey Lewis et al showed in 2011 that miR-122 was released earlier than ALT, the current gold standard, and that it was more sensitive also. (171) Recently, work from Rotterdam by van der Meer et al has also shown that miR-122 is more sensitive in detecting hepatotoxicity, in their case in patients with hepatitis C virus. (188) This seems to confirm the potential for miR-122 to have an important role in detecting hepatotoxicity in the future.

Despite ALT being the current gold standard biomarker for hepatotoxicity, it is known that it correlates poorly with histopathological damage. (168) A new biomarker that could predict the extent of histopathological damage would be beneficial in clinical practice. Antoine et al published work in patients who had taken an acute paracetamol overdose resulting in admission to a national liver transplant unit. They showed in patients with initially normal standard liver function tests that miR-122 and a panel of other novel biomarkers, could strongly predict those who went on to

develop liver injury and those who did not. (189) This is potentially very important as any biomarker, either on its own or as a panel of markers that could predict end liver damage would allow for a greater degree of personalized therapy, potentially improving outcomes for each individual patient.

The mechanism of release of miR-122 is still not well known. It is accepted that when the damage causes the cell wall to rupture, miR-122 along with other intracellular molecules such as ALT are released into the circulation. The earlier release of miR-122 suggests that another mechanism may be present. Bala et al showed that miR-122 is released from the cell in different ways. They showed that depending on the type of injury, it may be released either in exosomes or in a protein fraction. (183) Since this paper was published, other groups have suggested that there is indeed an active release of miR-122 from stressed cells. (182; 190) If there is active release of miR-122 at low levels of stress, this could explain the earlier release over ALT and could allow diagnosis of hepatotoxicity before it becomes severe enough that there is widespread necrosis and cell wall rupture.

With the increasing evidence that miR-122 is very specific and sensitive for liver damage, some groups have been investigating its role in monitoring response to therapy in various liver conditions. Koberle et al have investigated miR-122 levels in patients treated for HCV. They showed that miR-122 levels dropped after treatment. In those with sustained viral response, miR-122 stayed low, while in those in whom therapy failed, the levels returned to baseline. (191) Although this work was done in HCV, the potential to track the success of therapy has many possibilities. In particular relation to our work in colorectal liver metastases, it may be possible to track levels during chemotherapy and detect increasing hepatotoxicity as the treatment progresses.

5.3 What this thesis has added to the literature on miR-122 as a biomarker.

In order to understand abnormal, one must understand normal first. To date there has been no publication in the literature looking to understand the behaviour of serum miR-122 under normal conditions in either animal models or humans. We have looked at the serum levels of miR-122 in terms of both inter- and intra-patient variation in humans. We have shown that serum miR-122 is detectable in normal levels and that it is found within a relatively small range within the population. There is neither significant circadian variation nor longitudinal change over several days, meaning that sampling time should not affect the validity of the result.

Throughout the literature the method of expressing the data produced by the PCR process varies significantly as does the actual methodology. We have investigated the different methods of normalising and expressing data and shown the potential strengths and weaknesses. We have also developed a novel method to give relative quantification that can be adopted whatever methodology is used allowing for valid comparison between papers and results.

It is important that more baseline work is carried out if miR-122 is to become a mainstream biomarker. Further work would need to increase the numbers and expand the age range into the paediatric and elderly age ranges to firm up the defined normal range.

This thesis looked at healthy individuals with no past medical history or prescription medications. While this does represent a “healthy” cohort, it does not really represent a population “normal” cohort. In such a “normal” cohort there would be patients with a variety of medical conditions, those who take prescription drugs and those who smoke or drink alcohol. This would be a much more clinically relevant control population and it may be valuable to expand the work in this area.

Although there are a significant number of papers published looking at miR-122 in disease and injury, these nearly all focused on a single aetiology and the differing methods and data expression mean that comparison between them is not possible. We have compared within the same experiment a number of differing aetiologies' and have shown that there is a difference in the levels of miR-122 released into the circulation depending on the underlying disease. This has not been shown in the literature before and is an important aspect of the role of miR-122 that requires further investigation.

The patient samples available for this experiment were historical, retrospective samples for which detailed clinico-pathological data were not available. It would be preferable to design a prospective experiment along these lines, sampling patients with a wide variety of liver diseases and injuries but also collecting good quality clinical and pathological data on them to add weight to the subsequent analysis.

5.4 MicroRNA-122 as a biomarker for chemotherapy-induced hepatotoxicity

The evidence for a role for miR-122 in drug-induced hepatotoxicity is well documented in animal models and has been confirmed in one of the only human drug toxicity studies performed to date (171). It is important to note that in the human study, clinically significant and potentially fatal hepatotoxicity was seen in all patients following acute paracetamol overdose. This represents the extreme end of the spectrum of DILI whereas the pilot study described in this thesis was designed to look for much more subtle changes. Whilst many of the patients showed histologically observable perturbation, this was uniformly mild, and is unlikely to have had an impact on either the ability to complete the chemotherapy or the outcome.

Learning from this pilot study, there are a number of changes that could be incorporated into a larger study that may be beneficial in improving the validity of the results.

The first is the number of patients recruited. Despite only being a pilot study and therefore not being powered, it is clear that the low numbers recruited had an impact on the results and findings. By increasing the numbers, the chances of a false negative would decrease.

The second is the timing of the blood sampling. Patients within the pilot study had their blood samples taken prior to the administration of the next cycle of chemotherapy. Subsequent work from Starkey Lewis (unpublished) has shown that the half-life of miR-122 is short. (Figure 50) It may be that any acute rises in miR-122 would have occurred soon after the administration of the chemotherapy and that by the time the blood sample was taken the levels had returned to normal.

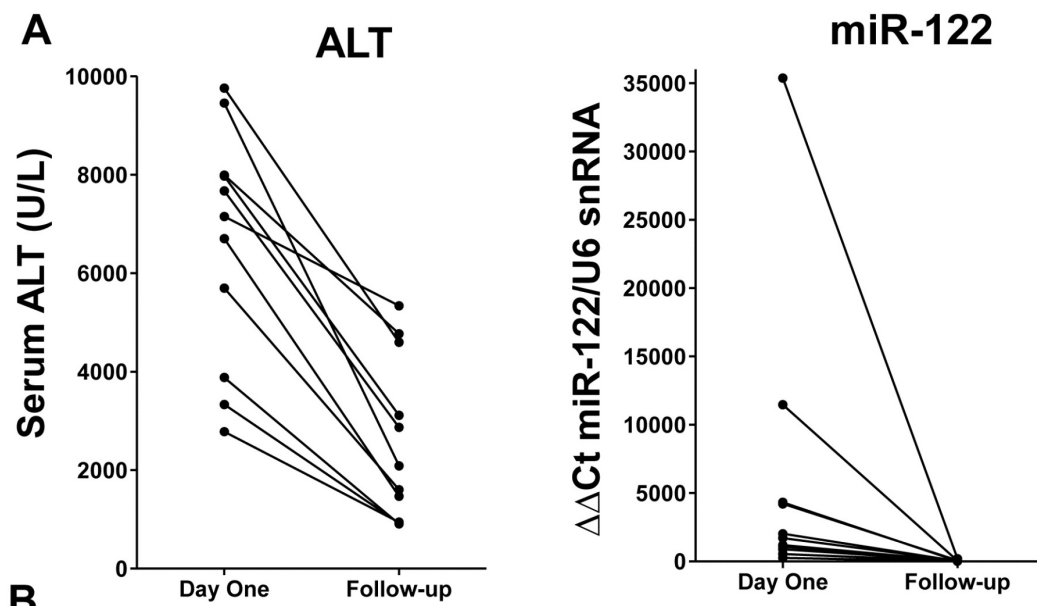


Figure 50: Unpublished data from Dr Philip Starkey Lewis of the MRC Centre for Drug Safety Science, University of Liverpool. This shows that miR-122 returns to normal levels quickly compared to ALT after hepatotoxicity caused by acute paracetamol overdose.

Ideally samples should be collected both immediately pre- and post-administration of the chemotherapy (up to 24hrs post) rather than only pre-delivery. This could allow acute rises in miR-122 to be detected following

administration of the drug. This would involve new ethical approval as it would mean an extra blood sample that is not part of standard care (post chemo sample) and this may also impact upon compliance.

The study would also greatly benefit from the inclusion of a pre-chemotherapy liver biopsy to allow direct comparison with the resected post-chemotherapy specimen. This would allow a controlled assessment of any liver damage in the latter biopsy, and the assignment of chemotherapy-associated toxicity. This is a major limitation of the pilot study as it is at present. The delay between finishing chemotherapy and having surgery is also a confounding variable as any potential liver regeneration cannot be taken into account. It is of course unlikely that it would be ethically appropriate to perform a pre-chemotherapy liver biopsy as the risks of such a procedure are significant. To this extent the development of animal models of chemotherapy-induced hepatotoxicity hold great promise (192) as they can allow the pre- and post-chemotherapy histology that would be extremely valuable information in assessing the role of miR-122.

5.5 Chemotherapy for Colorectal Liver Metastases

Recently there have been significant developments in this field. Good quality evidence is particularly hard to develop in clinical medicine as technology is developing so quickly that long-term follow-up studies are old before they complete. The landmark study when I started this thesis was the European-wide EPOC trial looking at the role of neo-adjuvant chemotherapy in colorectal liver metastases. (56) It was hoped that this study would provide the evidence for the role of neo-adjuvant chemotherapy that many experts thought was logical in the treatment of these patients and indeed early data suggested a longer progression free survival in the chemotherapy group, seemingly vindicating the case for chemotherapy.

This outcome was challenged and the case for chemotherapy revisited in 2012, when at the annual meeting of the American Society for Clinical Oncology (ASCO), the first longterm data from the trial was presented suggesting no overall survival benefit occurred from the use of

chemotherapy but it was associated with more side-effects. The data has since been published confirming the lack of overall survival benefit. At a median follow-up of 8.5 years the hazard ratio was 0.88 (95% CI 0.68-1.14, $p=0.34$). (187)

This seemed to suggest there was no role for neo-adjuvant chemotherapy in patients with resectable disease. The authors however, state that given that PFS was the primary endpoint and that OS was an under-powered secondary endpoint, that these patients should still receive chemotherapy based on the increased PFS shown in the original paper. At present, based on the actions of the SMDT at University Hospital Aintree, the use of chemotherapy is only now used selectively with most patients with resectable disease proceeding directly to resection.

The potential benefit of biological agents has been shown in studies relating to irresectable disease. (69; 71-73) Given the excellent response rates seen, a role for biological agents in resectable disease was hypothesised. The New EPOC trial, sought to develop the evidence for cetuximab in patients with resectable disease. Patients were given FOLFOX/FOLFIRI +/- cetuximab in a randomised phase III trial that closed after 100% recruitment of its intended total. Surprisingly the study had to be stopped early after it met a pre-defined futility analysis. Early analysis showed significantly worse PFS in the cetuximab arm ((14.8 vs 24.2 months, HR 1.50037 (95% CI 1.000707-2.249517), $p<0.048$). Outcome in the FOLFIRI arm was also worse with cetuximab ((15.2 vs 24.2 months, HR 1.565546 (95% CI 1.014967-2.414793), $p<0.043$). This data was presented at the ASCO Annual Meeting in 2013 ((J Clin Oncol 31, 2013 (sup; abstract 3504)). Full publication of these data is still awaited, but the data presented in the abstract suggests there is no role for cetuximab in patients who are KRAS wild type and have resectable liver metastases.

The third trial under the EPOC umbrella was the EPOC B trial attempting to answer the question of timing of chemotherapy. This was a pilot, feasibility study randomizing patients to pre- or post-operative chemotherapy.

Unfortunately, the trial struggled with recruitment and was closed prematurely with only 25% of the intended numbers.

These three trials highlight the difficulties faced in producing high quality evidence in clinical medicine. Currently the use of chemotherapy in patients with colorectal liver metastases remains controversial. There is general agreement that it is indicated in the initially unresectable or borderline patient, and the evidence for this is well founded and accepted. (59; 61; 62) The debate about its role in resectable disease continues. Some quote the futility of the EPOC family of trials as a reason not to give chemotherapy, while the advocates of chemotherapy point to the limitations of the trial as a reason to reject the findings. A systematic review carried out by Primrose et al in 2006 showed that up to 61.3% of patients who had a liver resection developed recurrent disease but that in 65% of them the recurrent disease was extra-hepatic in nature. This adds weight to the argument for chemotherapy by showing that resectional surgery, which is organ specific, does not deal with the systemic nature of the disease for which the chemotherapy is necessary.

As new chemotherapeutics are developed and new combinations trialed, the role for chemotherapy is likely to increase. Despite the often negative outcomes of trials, advanced colorectal cancer is a systemic disease and can only be treated by systemic therapy.

5.6 Summary and review of hypotheses.

The first rule of medicine is “do no harm”. Chemotherapy has side-effects and it is being increasingly recognised that the “one size fits all” dogma is not the way forward and that personalised therapy is the future. In patients for whom it is felt that there is a potential benefit of chemotherapy, undiagnosed side-effects may have an overall negative outcome for them. The need to have robust biomarkers of toxicity to allow alterations in therapy on an individual patient basis remains a key focus of future

research. To this end we set out to explore the potential role of microRNA-122 in the early detection of chemotherapy-induced hepatotoxicity in patients receiving neo-adjuvant chemotherapy for the treatment of advanced colorectal cancer.

We would revisit the hypotheses we set out to investigate;

- That miR-122 is detectable in the serum of healthy human subjects.

Proved. We were able to detect miR-122 in the serum of healthy volunteers.

- That miR-122 exists within a narrow range in the normal population and does not vary with time.

Proved. We showed that serum miR-122 had low variation and did not change in a circadian rhythm or over the course of several days.

- Patients with liver disease/injury will have higher levels of miR-122 than a normal population.

Proved. We showed that there was a significantly raised serum miR-122 in patients with liver disease compared to normal subjects.

- MiR-122 will be more sensitive than serum transaminases in liver disease and injury.

Proved. We showed that miR-122 was better at diagnosing injury than serum AST in patients with inflammatory changes.

- MiR-122 may be of use in diagnosing hepatocellular carcinoma (HCC) within an at-risk patient group.

Not proved. There was no difference in the levels of serum miR-122 between the HCC and non-HCC patients.

- That miR-122 can detect chemotherapy induced liver injury in patients undergoing neo-adjuvant chemotherapy for colorectal cancer liver metastases.

Not proved. We were unable to show a relationship between serum miR-122 and hepatotoxicity.

- That miR-122 is more sensitive and specific than transaminases at predicting chemotherapy induced liver injury.

Not proved. We were unable to show a relationship between serum miR-122 and hepatotoxicity.

Over the course of this thesis believe that we have shown that miR-122 may have a role in this field, but that more work is required to confirm it and develop the field further, particularly in the area of chemotherapy-induced hepatotoxicity.

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